ABSTRACT

Delia W. Johnson. (Under the direction of Ralph J.Smialowicz, PhD.)

SENSITIVITY OF THE SRBC PFC ASSAY VS ELISA FOR DETECTION OF IMMUNOSUPPRESSION BY TCDD AND TCDD-LIKE CONGENERS.

The splenic antibody plaque-forming cell (PFC) assay to sheep red blood cells (SRBC) is considered to be one of the most sensitive assays for detection of immunosuppression, and traditionally has been used in immunotoxicity testing. However, an enzyme-linked immunosorbent assay (ELISA) (Temple et al., 1993), is gaining popularity as a more convenient and less expensive alternative. The PFC assay quantifies the number of anti-SRBC producing plasma cells in the spleen, while the ELISA measures SRBC-specific IgM antibody in the serum. This study was performed to compare the sensitivity of these two assays to detect immunosuppression by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and TCDD-like congeners. Two sets of 6-7 groups of female B6C3F$_1$ mice (6-7 mice/group, 8-weeks-old) were given a single oral exposure to corn oil or different doses of TCDD or congeners. Seven days later, mice were immunized i.v. with SRBC. The first set of mice was evaluated by the PFC assay and the second by the ELISA, on day 4 or 5 post-immunization, respectively. The four TCDD congeners tested were: 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin (PeCDD), 1,2,3,4,7,-pentachlorodibenzofuran (4PeCDF), 3,3',4,4',5-pentachlorobiphenyl (PCB126) and 2,3',4,4',5-pentachlorobiphenyl (PCB118). The ED_{50s} for TCDD and each congener were determined from the PFC and ELISA data. For all the chemicals tested the ED_{50s} for the ELISA were lower than the ED_{50s} for the PFC, with percent differences ranging from 33 to 84%. These results indicate that the ELISA is a more sensitive assay for detecting suppression of the SRBC-specific IgM response of dioxin-like chemicals in this rodent model and supports its use as a sensitive alternative to the PFC assay.

ACKNOWLEDGMENTS

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TABLE OF CONTENTS

LIST OF TABLES

List of Figures

1.0 INTRODUCTION

The immune system is a complex web of organs, cells, and soluble factors which act together to preserve host integrity. When functioning optimally, the immune system must recognize subtle differences between "self" and "non-self". Because it operates within such a narrow range, exposure to certain chemicals can result in seemingly subtle immunological changes that detrimentally impact normal operation of the immune system. An immune response which errs in the recognition of "self" may injure the host's own tissues, resulting in autoimmune diseases. By the same token, an inappropriate potentiation or enlargement of an immune response can lead to allergy or hypersensitivity. On the other hand, an impaired or suppressed immune system may fail to recognize foreign agents (e.g., viruses, bacteria, parasites) in time to prevent severe infection. Transformed neoplastic cells may not be identified as "non-self" before tissue damage occurs and the spread of cancer threatens.

Immunotoxicity refers to "the ability of a test substance to suppress immune responses that could enhance the risk of infectious or neoplastic disease, or to induce inappropriate stimulation of the immune system, thus contributing to allergic or immune disease" (EPA Health Effect Guidelines, Immunotoxicity). Due to the complexity of the immune system, a battery of immune assays has arisen to screen for potential compounds that are immunosupppressive. These tests have been organized into "tiers" to test for functional and pathological immune parameters by the National Toxicology Program (NTP). The NTP developed standard protocols for the immune assays included in the screening battery (Luster et al., 1988). Selected assays were tested in interlaboratory trials for reproducibility, sensitivity and predictability with a number of known immunotoxicants. Investigators later compared tests to find a simple screening method to accurately identify potentially immunotoxic compounds. Analysis indicated that two or three tests were sufficient to predict immunotoxicity in rodent models. The test demonstrating the highest

association with immunotoxicity was the splenic antibody plaque-forming cell (PFC) assay which measures the antibody response to sheep red blood cells (SRBCs) (Luster, et al. 1988).

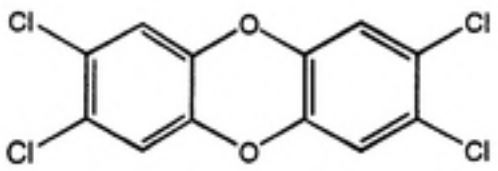
Accordingly, the PFC assay is widely used and is included in the Federal Insecticide, Fungicide and Rodentide (FIFRA) guidelines established by the EPA's Office of Pesticides Program (OPP) to assess chemicals for immunosuppressive potential. A recent revision of the OPP health effects test guidelines for immunotoxicity recommended that either the PFC response or the SRBC-specific serum IgM enzyme-linked immunosorbent assay (ELISA) be used to assess the primary humoral response to SRBCs (USEPA Health Effects Test Guidelines, Immunotoxicity, 1998). Either of these assays may be conducted in animals on standard toxicology study without altering standard toxicology endpoints (Ladics, et al., 1998).

Because these two assays measure different endpoints, there is a need for comparison of sensitivity and reliability. The PFC assay quantifies the number of plasma cells in the spleen producing SRBC-specific antibody, while the ELISA measures SRBC-specific IgM antibody in the serum. There are several advantages to the SRBC ELISA. It can be processed by one technician in four hours, while the PFC assay requires a minimum of two to four technicians and requires a full day's work. The ELISA is more cost-effective. For example, the animals need not be euthanized, since only a blood sample is required, and could be used for other types of toxicology assessment. Also, test sera can be stored for later analysis. Potentially, the ELISA could be developed as a commercially available kit, which would make for more uniform results among testing laboratories.

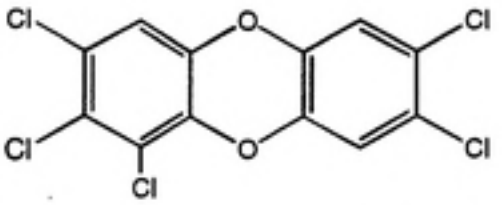
The purpose of this project was to determine if these two assays are equally sensitive to suppression of the SRBC response in a rodent model. The project was divided into four phases. The first phase of the project focused on optimizing conditions for the SRBC IgM specific ELISA as developed by Temple et al. (1993) and for the SRBC PFC protocol currently used in this laboratory using B6C3F1 female mice and Sprague-Dawley male rats. During the second phase the time, in days, for optimal PFC and antibody production determined by ELISA was assessed following a single intravenous (iv) injection of SRBCs in both rodent models. In the third phase, the sensitivity and comparability of the ELISA technique relative to that of the PFC assay was explored with known immunosuppressive

halogenated aromatic hydrocarbon (HAH) chemicals.

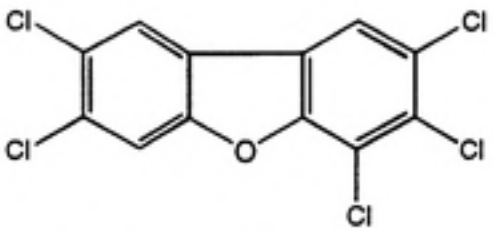
2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) is considered to be the most thoroughly examined xenobiotic which has been evaluated as an immunotoxicant (Holsapple, 1991). Therefore, TCDD and TCDD-like congeners were logical choices for test chemicals. The four TCDD congeners tested were: 1,2,3,7,8-pentachlorodibenzo-p-dioxin (PeCDD), 1,2,3,4,7,-pentachlorodibenzofuran (4PeCDF), 3,3',4,4',5-pentochlorobiphenyl (PCB126) and 2,3',4,4',5-pentachlorobiphenyl (PCB118). The molecular structure of these test chemicals is shown in Figure 1. A single oral exposure of female B6C3F1 mice to different doses of TCDD or TCDD- like congeners was followed by immunization with SRBCs. In the fourth phase of the project, the results were used to generate dose-response curves for TCDD and each TCDD congener.With a computer model, ED_{50} values and dioxin immunotoxicity relative potency factors (REPs) were estimated from the data from both assays for all test chemicals.



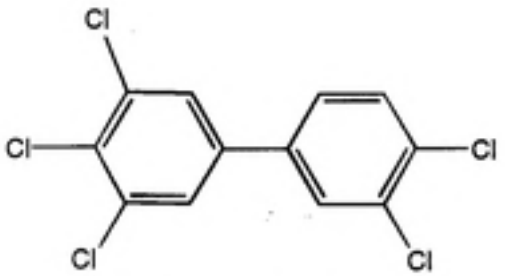
2,3,7,8-TETRACHLORODIBENZO-p-DIOXIN
(TCDD)



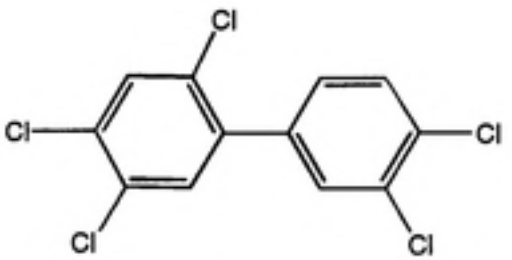
1,2,3,7,8-PENTACHLORODIBENZO-p-DIOXIN
(PeCDD)



2,3,4,7,8-PENTACHLORODIBENZO-p-FURAN
(4PeCDF)



3,3',4,4',5-PENTACHLOROBIPHENYL
(PCB126)



2,3',4,4',5-PENTACHLOROBIPHENYL
(PCB118)

Figure 1. Molecular structure of test chemicals.

1.1 IMMUNOLOGY REVIEW

General references for this section:

Janeway, C. A., and Travers, P. ***Immunobiology: The immune system in health and disease.*** © 1994. New York: Current Biology, Ltd./ Garland Publishing, Inc.

Paul, W.E. (Ed). ***Fundamental Immunology, 3rd Edition.*** © 1993. New York: Raven Press, Ltd.

Roitt, I., Brostoff, J., and Male, D. ***Immunology.*** ©1985. London: Gower Medical Publishing, Inc.

Wier, D.M. (Ed.) ***Handbook of Experimental Immunology, 3rd Edition***. ©1978. London: Blackwell Scientific Publications.

The immune system has several responses to recognize and incapacitate an invader. Distinct cell types cooperate in many responses and individual cells may perform more than one function. The immune response is divided into two functional divisions, namely innate immunity and acquired immunity. Innate immunity is a non-specific response: it does not require prior exposure to a "non-self" antigen to operate, and lacks specificity for any particular antigen. Cell types involved in this response include phagocytes (i.e. macrophages and polymorphonuclear leukocytes) which engulf and kill certain infectious agents (i.e. bacteria, fungi, parasites and viruses), and natural killer cells which possess cytolytic activity. Soluble factors, such as the complement protein system and cytokines are also involved. Innate immunity holds infection at bay until the acquired immune system can take over.

Acquired immunity is an antigen specific system. Prior exposure to antigen is required to provoke the most effective defense. The acquired immune system is said to "remember" a particular antigen recognized on the pathogen and can more quickly prevent the pathogen from causing disease later. Acquired immunity includes both cell- mediated and humoral immune responses. Originally, the term cell-mediated immunity was used to refer to

reactions to intracellular pathogens involving T cells and phagocytes, rather than B cells and antibody. It is now known that cell mediated and antibody-mediated responses may overlap. The term cell-mediated immune response is now used to describe a response where antibody production plays a subordinate role. Humoral immunity takes its name from the fact that bodily fluids were known as "humors". Protection from certain pathogens could be achieved by taking "humor" from an immunized host and transferring it to a naive host. Today, scientists know that the observed protection in the naive animal is due to the antibodies in the host serum.

Only B lymphocytes have the potential to make antibodies. B cells begin as stem cells in the bone marrow. In this environment, they mature and acquire tolerance to self-antigens. The B cells that recognize self-antigen in the bone marrow are selectively destroyed, thereby removing self-reactive cells and leaving naive B cells. Naive B cells , or B cells which have not encountered foreign antigen, leave the bone marrow and enter circulation. Naive T cells develop in the thymus and enter circulation upon maturation. In secondary lymphoid organs such as the lymph nodes and spleen, naive B cells encounter trapped foreign antigen as well as T cells and antigen-presenting cells. The antigen-specific B cells proliferate and mature into plasma cells which are capable of producing antibody specific to antigen. Some plasma cells remain in the lymphoid organ and others migrate to other lymph tissues and back to the bone marrow. In mammals, the bone marrow is the major site of antibody production in secondary immune responses (Benner et al., 1981).

The basic structure of all immunoglobulins is a unit consisting of two identical light polypeptide chains and two identical heavy polypeptide chains linked together by disulfide bonds. Light chains exist in two configurations referred to as ***kappa*** and ***lambda***. Different heavy chains determine the antibody classes. Furthermore, each antibody molecule can be divided into a "constant" and a "variable" region . The constant region is not involved in antigen binding but is recognized by receptors located on the surface of many immune cells. The variable or antigen-binding region is located on both heavy and light chains. This section has significant sequence variability, and is specific for an antigen.

An antibody may be characterized by its binding properties to antigen. The strength of a single antigen-antibody bond is termed antibody affinity. This is the sum of all forces,

attractive and repulsive, between one variable region of the antibody and the antigen. The overall strength of binding of an antibody molecule to an antigen is called its avidity. For example, an antibody reacting with the surface antigen of a pathogen will often bind the same molecule or particle with both of its binding sites, increasing avidity.

The five antibody classes (e.g., isotypes) are distinguished by type of heavy chain and are IgA, IgD, IgE, IgG and IgM. IgM antibodies are the first class to appear in the primary response and are confined to the circulatory system. The molecule is a polymer made up of repeating units containing the basic structure of all immunoglobulins: two heavy chains and two light chains. An additional polypeptide chain, the J (joining) chain acts to assist polymerization of the separate units prior to secretion by B cells.

Upon exposure to antigen, the primary antibody response is elicited, which consists of 4 phases:

1. Initial **lag** phase: no antibody can be detected.
2. **Log** phase: antibody titer rises logarithmically.
3. **Plateau** phase: antibody titer stabilizes.
4. **Decline** phase: antibodies are catabolized, or bind to antigen and are cleared from circulation.

IgM antibodies are the first antibodies produced, and form a major proportion of the primary response. When animals are rechallenged with the same antigen, the secondary response will differ significantly. Specifically, IgG is the major component of the secondary response, and reaches a greater plateau than IgM which remains constant. The secondary response begins more rapidly and persists longer than the primary response.

The immune response to a particular antigen may be elicited by B cells alone (T cell-independent), or may require cooperation by T cells, B cells, and antigen presenting cells (T cell- dependent). This distinction is used in immunotoxicology studies designed to determine immune cell types affected by a xenobiotic. Only a few antigens, such as lipopolysaccaride (LPS) a component of bacterial cell walls, are T cell-independent antigens. T cell-dependant antigens, like SRBCs require many immune cell types for antigen clearance. The immune response can be initiated in any of the peripheral immune organs.

Peripheral lymphoid organs are specialized to trap antigen from different body

compartments, and vary in appearance and size. The spleen is specialized to trap antigen circulating in the blood. It consists of red pulp, the site of aged erythrocyte destruction, interspersed with lymphoid white pulp. Lymph nodes trap antigen in lymphatic fluids from tissues, while the tonsils and Peyer's patches collect antigen from the epithelial surfaces of the gastro-intestinal tract.

Despite these differences, secondary lymphoid organs share the same basic architecture. Areas where B cells are located are called follicles or the B cell corona and are next to T cell areas. Germinal centers are specialized sites found at the junction between the B and T cell areas. In this area, B cells specific for antigen proliferate and differentiate into antibody secreting plasma cells. These mature B cells can reenter circulation and deposit in other lymph tissue.

1.2 Review of 2,3,7,8 Tetrachlorodibenzo-p-dioxin

The compound 2,3,7,8 tetrachlorodibenzo-p-dioxin, also referred to as TCDD or "dioxin", is the most toxic of a group of compounds known as halogenated aromatic hydrocarbons (HAHs). Included in this group of chemicals are the polychlorinated dibenzofurans (PCDFs), polychlorinated dibenzodioxins (PCDDs), and polychlorinated biphenols (PCBs). Members of this family of compounds exhibit common properties. They tend to be highly lipophilic with the degree of lipophilicity increasing with greater ring chlorination. Also, many are very resistant to breakdown both environmentally and biologically.

In the environment, dioxin is never found alone but exists in a mixture of congeners. In fact, many of the other HAHs are present in the environment in much greater amounts than TCDD (Birnbaum and DeVito, 1995). However, scientific and regulatory interest has focused on TCDD because it is the most toxic of these compounds. The bulk of available toxicological information concerns TCDD, but there is limited information on many TCDD congeners.

TCDD was never produced intentionally, rather it was formed as unwanted industrial byproducts of industrial and combustion processes. Examples of such processes are the synthesis of compounds such as the herbicide 2,4,5 trichlorophenyoxyacetic acid (the active ingredient in Agent Orange), hazardous waste incineration, and the bleaching of paper and wood pulp. Although dioxin persists in the environment, it breaks down quickly in sunlight if a proton donor (e.g. plant material or oils) is available for photolysis (Tschirley, 1986). Like many compounds of its class, dioxin is resistant to physical, chemical and biological degradation. Consequently, TCDD has a long half-life in animals and man and bioaccumulates up the food chain. The half life of TCDD in humans, for example, has been estimated to range from 7-11 years. Due to accumulation of TCDD in the food chain, the

primary route of exposure for 90% of the general population is from the diet, especially from meat, fish, and dairy products (Gilman and Newhook, 1991). Current daily dietary intake of PCDDs and PCDFs in US foods has been estimated as 2.0-4.2 pg TCDD TEQs/kg body weight/ day for adults (Gilman et al.,1997) with a body burden estimate for the general population in the range of 5-10 ppt lipid adjusted. (Scheter and Olson, 1997). These values are similar to those reported in Canada, Germany, England and the Netherlands. Once TCDD has been absorbed, it readily distributes to all organs, with the highest concentration present in the adipose tissue (Poiger and Schlatter, 1986).

Studies beginning in the early 70's revealed that TCDD was an extremely potent toxicant in laboratory animals including, but not limited to fish, birds, rats, mice, guinea pigs, hamsters rabbits, dogs, and non-human primates. The LD50 has been reported as low as 0.6 μg/kg in guinea pigs (McConnell et al., 1978) and 25 μg/kg in rats (Beatty et al., 1978). Several studies using different animal models determined that TCDD is a multisite carcinogen in many species in both sexes, and is a potent tumor promoter. Further animal studies have presented evidence for a myriad of noncancer health effects, including endocrine toxicity, developmental toxicity, immunotoxicity, neurotoxicity and reproductive toxicity. Numerous experimental findings have pointed to the role of TCDD as a growth disregulator. Dioxin has been shown to influence a wide range of biochemical pathways, including hormones, inflammation factors, interleukins, growth factors, metabolic activation pathways, and protooncogene expression. Due to expanding knowledge about growth factors and dioxin's influence on cell proliferation rates, interest is growing in noncancer endpoints and the mechanism of action of these effects.

Evidence of cancer and noncancer health effects after exposure to TCDD in humans has been limited and inconclusive. Documented human health effects have included porphyria, altered glucose metabolism and increased risk of diabetes in highly exposed individuals, hormonal effects (altered thyroid hormone and testosterone levels), and reproductive effects (Roegner et al., 1991, Calvert et al., 1999, Egeland et al., 1994). A skin

condition, chloroacne, has been the most consistently reported malady in humans after massive exposure to TCDD (Mocarelli et al., 1991).

1.3 Immunotoxic Effects of TCDD

Of the many organs/systems affected by TCDD, one of the most sensitive is the immune system. Consequently, TCDD is considered to be the most thoroughly examined xenobiotic which has been evaluated as an immunotoxicant (Holsapple, 1991). Exposure to TCDD results in thymic atrophy, alterations in bone marrow, and general immunosuppression in almost every species examined (Luster 1979, 1980). Both cell-mediated and humoral responses are suppressed following exposure to TCDD, suggesting multiple cellular targets within the immune system (Kervliet, 1995, Vos et al., 1997). Numerous host-resistance studies indicate that exposure to TCDD results in increased susceptibility to bacterial (Thigpen et al., 1975), viral (Burleson et al., 1996, House, RV et al. 1990), parasitic (Luebke et al., 1994) and neoplastic disease (Luster et al., 1980).

An effect of sublethal exposures of TCDD common to all species examined is thymic atrophy, which has been shown to be dose-dependant (Vos et al., 1973). For example, following a single dose of TCDD, acute ED_{50} values for thymic atrophy were calculated as 26 μg/kg in Sprague Dawley rats, 0.8 μg/kg in guinea pigs, and 48 μg/kg in Syrian golden hamsters (Hanberg et al., 1989). Thymic atrophy was observed in rhesus monkeys after a single dose of 70 μg TCDD/kg (McConnell et al., 1978), and in rats after a single administration of 1 μg TCDD/kg (De Heer et al., 1994). An intact thymus is crucial to the developing immune system during the prenatal and postnatal period of rodents as well as humans. In very young animals, congenital thymic aplasia or thymectomy severely reduces the number and function of T cells (Benjamini and Leskowitz, 1991). However, in adult animal models, removal of the thymus has been shown to have little or no effect on the quantity or quality of T cells (Benjamini and Leskowitz, 1991). By this stage, T cells have already matured and moved to secondary lymphoid organs. Suppression of immune responses in adult animals occurs at levels of TCDD and related congeners significantly

lower than those needed to induce thymic atrophy.

Experimental evidence suggests that very young animals are more susceptible to HAH immunotoxic effects of TCDD (Birnbaum et al., 1995). Suppression of T cell mediated immunity in rodents occurs at lower doses of TCDD when the animals are exposed prenatally (Holladay, et al., 1991, Vos and Moore, 1974). For example, a single dose of TCDD (3 μg/kg) to pregnant F344 rats resulted in thymic atrophy and alteration of T cell development in neonates, and pups (Gehrs et al., 1997). Suppression of the delayed-type hypersensitivity response in these offspring persisted into adulthood and was associated with an even lower dose (0.1 μg/kg) to the dam (Gehrs et al., 1999). Given the weight of evidence that the thymus is a target organ, it is not surprising that alterations in T cell responses occur upon exposure to TCDD. Delayed -type hypersensitivity (DTH) , cytotoxic T lymphocyte (CTL) activity, helper T cell function, and T cell-dependant antibody responses, such as the response to SRBC, have been shown to be suppressed upon exposure to TCDD *in vivo* (Vos et al., 1973, Kervliet et al., 1990, Tomar and Kervliet, 1991, De Krey et al., 1995).

TCDD-mediated suppression of humoral immunity has been most frequently examined by the primary PFC antibody response to SRBCs. This is also one of the most sensitive and reproducible immune endpoints suppressed by TCDD and TCDD-like congeners. (Kervliet et al., 1993). An ED_{50} of approximately 1.0 μg TCDD/kg for TCDD-induced suppression of the anti-SRBC response in adult B6 or B6C3F1 mice has been consistently reported by several laboratories (Davis and Safe, 1988, Smialowicz et al., 1994). Antigens that differ in their requirements for antigen presenting cells (APCs) or T cells can be used to evaluate cellular targets for immunotoxicity. B cells alone cannot mount any kind of an antibody response to SRBC, a T cell-dependent antigen. TNP-LPS is a T cell independent antigen, therefore adequate B cell function is needed for a robust TNP-LPS response. The differential sensitivity of the antibody responses to TNP-LPS versus SRBC has been shown in TCDD-treated mice (House et al., 1990) and rats (Smialowicz, 1996). Treatment with TCDD depressed both responses, but suppression of the response to SRBC was achieved with a lower dose of TCDD. This would suggest that TCDD targets the T cell and/or APC components more so than the B cell response.

By contrast, although direct effects of TCDD and other HAHs on T cells *in vitro* has

not been demonstrated, *in vitro* studies have shown direct effects of these chemicals on the activation and differentiation of purified B cells (Tucker et al., 1986, Luster et al., 1991). An *ex vivo* experiment in which the investigators removed spleen T cells, B cells and macrophages from TCDD and vehicle treated mice found B cells from TCDD-treated mice were functionally compromised, but T cells and macrophages were not (Dooley and Holsapple, 1988).

As it can be seen, due to the complexity and overlapping nature of the immune response it is difficult to tease out which cellular components of the immune response are targeted and the mechanism by which they are impaired by TCDD and TCDD-like congeners. Despite the extensive body of work accumulated thus far on the immunotoxic effects of TCDD, scientific investigation is far from complete.

1.4 Role of the Ah Receptor in TCDD Mediated Toxicity

The initiation of the majority of dioxin-induced effects are mediated by the Ah receptor, which functions as a ligand-activated transcription factor. The existence of an induction receptor for TCDD and related HAHs was first postulated by Poland and Glover in 1973. The receptor, termed the Ah (aromatic hydrocarbon) receptor, was first identified in the cytosol of the mouse liver (Poland et al., 1976). Over time, the Ah receptor was found to be ubiquitous in tissues and organs of mammalian and non-mammalian species, in human tissue and in human cell-lines (Van Den Berg, 1998). A series of experiments examined the structure-binding relationship for a number of TCDD congeners in the mouse hepatic cytosol (Poland et al., 1979). The affinity of the congener for the Ah receptor was determined by the number of substituted chlorine atoms. TCDD, the most active chemical of this group, has chlorines in all four lateral positions. The addition or removal of lateral chlorine substituents results in congeners with lower Ah receptor binding affinities.

The Ah receptor is a protein complex consisting of two heat shock protein molecules (HSP90) among other peptide molecules. Prior to occupancy by a ligand, the inactive Ah receptor resides in the cytoplasm. It appears that the HSP90 maintains the Ah receptor in a ligand-binding conformation and represses it's DNA binding capacity (Okey et al., 1994). The binding of TCDD or TCDD-like congeners to this receptor is reversible and occurs with high affinity. Once TCDD is bound to the receptor, a conformational change occurs releasing the two heat shock proteins. Upon heterodimerization with another protein, the Ah receptor nuclear translocator (ARNT) protein the entire complex migrates to the nucleus where it can induce the transcription of genes by binding to specific "dioxin responsive" elements (DRE). Conclusive evidence indicates that the CYP1A1 gene (responsible for production of cytochrome P4501A1) is induced through this pathway. *In vitro* induction of the CYP1A1 gene is used as a way to screen chemicals for dioxin-like activity (Harper et al., 1995).

Effects of TCDD and TCDD-like congeners on Ah responsive, and less responsive strains of mice suggest that TCDD-induced immunosuppression is mediated by the Ah receptor (Harper, et al.,1993, 1994). As the Ah locus codes for the Ah receptor, strains of mice that differ at the Ah locus also differ in their response to TCDD. While C57Bl/6 mice (Ah^{bb}) are highly responsive to TCDD-induced immunosuppression, DBA/2 (Ah^{dd}) mice are more resistant. DBA/2 mice require a ten-fold higher dose of TCDD, a six-fold higher dose of PCB 126 and a three-fold higher dose of PCB 169 compared to C57Bl/6 to achieve comparable suppression of the PFC response (Davis and Safe, 1990, Harper et al., 1994). This difference in strain sensitivity has been demonstrated for other immune endpoints affected by TCDD and related compounds, such as suppression of cytotoxic T lymphocyte activity (Kerkvleit et al. 1990). Furthermore, several studies indicate that there is a correlation between structure-immunotoxicity and Ah receptor binding affinities for TCDD and TCDD-like compounds (Clark et al., 1983, Davis and Safe, 1988, 1990, Kerkvliet et al., 1990).

These observations support a role for the Ah receptor in mediating the immunosuppressive effects of TCDD and similar HAHs. However, much work is still needed to clarify scientific understanding of the mechanism(s) involved in Ah-mediated immunotoxicity. Additionally, due to the interaction between the immune systems and other organs/systems (e.g. the endocrine system), it is possible if not likely that non-Ah receptor mediated events are also involved in HAH immunotoxicity.

1.5 Human Studies and TCDD- Mediated Immunotoxicity

Although a plethora of evidence has established TCDD as an immunotoxicant in animals, epidemiologic studies have not revealed consistent patterns of damage to the immune system by TCDD and its congeners. However, a few epidemiologic studies of humans exposed to TCDD through industrial accidents suggest subtle changes in the immune system and increased disease susceptibility in highly exposed populations. For example, TCDD-exposed workers were evaluated several years after a chemical plant incident in 1953 in Ludwigshafen, Germany. The proportions of some lymphocyte populations (B cells, T cells, T helper cells, and T suppressor cells) were lower among these workers when compared to an unexposed referent population (Ott et al., 1993). An increase in total lymphocyte count, serum IgG and IgA levels, incidence of appendicitis, thyroid disease, upper respiratory infections, and infectious and parasitic diseases was found in later studies (Ott et al., 1994, Zober et al., 1994). In central Taiwan during 1978-79, rice oil was contaminated with polychlorinated biphenyls and polychlorinated dibenzofurans. Adults exposed to the toxic chemicals exhibited decreased serum IgA and IgM levels, and a reduced helper/suppressor T cell ratio (Lu and Wu, 1985).

Very young animals are exquisitely sensitive to the immunotoxicity of dioxin and dioxin-like compounds. Prenatal and postnatal exposure to TCDD has been shown to result in immune alterations that persist into adulthood in F344 rats (Gehrs et al., 1999). Some studies propose that this may hold true for young humans. Results from a study on Dutch infants suggest that prenatal background levels of PCB/dioxin exposure influences the human fetal and neonatal immune system (Weiglas-Kuperus et al.,1995). A follow up study indicates that pre and postnatal background exposure is associated with changes in the T- cell population, the humoral response, as well as prevalence of chickenpox and middle ear infections in these children at 42 months of age. (Weisglas-Kuperus et al., paper submitted). Children from central Taiwan whose mothers had consumed the contaminated oil were exposed prenatally to polychlorinated biphenyls and polychlorinated dibenzofurans. Mothers of these children reported that they had an increased incidence in pneumonia and bronchitis in the first six months after birth (Rogan et al., 1988). A higher incidence of middle ear

diseases than controls was found in schoolchildren whose mothers were exposed to the contaminated rice oil. The authors stated that, compared to controls, "collectively these children either contracted otitis media easily or had more difficulty clearing the infection."(Chao et al., 1997).

Several explanations account for the lack of corroboration between human epidemiolgic studies and conclusive immunotoxic effects reported in animal studies. First, the assessment of subclinical changes in immune function in humans is limited. Human immune assays currently in use have a broad range of normal responses. Although marginal alterations in the immune response may be detected, the real impact on the function of the immune system may be insignificant or unknown. Secondly, researchers are restricted to assays that are clinically feasible versus those that are shown to be most sensitive in animals studies (Van Loveren et al., 1995). Obviously, ethics prevent certain testing schemes in human studies, such as challenge with infectious disease. Also, most epidemiological studies occur long after initial exposure to TCDD has occurred, and it is possible that at the time of the study subjects have recovered from the immunotoxicological effects of TCDD and TCDD-like congeners. Additionally, there are a number of confounding variables that influence the immune response, such as: age, sex, pregnancy, smoking , alcohol use, diet, sleep disturbances, illness, physical exertion, depression, acute stress, malnutrition, commonly used medicines, and certain minerals and vitamins (Janeway and Travers, 1994). Finally, TCDD and TCDD congeners are ubiquitous in the environment. Therefore, it is difficult, if not impossible, to define a "non-exposed" population as we are all exposed to background levels of TCDD and TCDD congeners.

As mentioned above, the immune response of animals exposed to HAHs is often assessed after challenge with an antigen or pathogen of interest. Several recent studies have been designed to examine the immune response to challenge (via vaccination) in humans exposed to HAHs. Continued improvements in immunologic tests used in human studies are needed to detect subtle changes in the immune response. Improvements in the researcher's ability to accurately determine human body burden of HAHs at the time of study, as well as predict initial exposure levels are necessary. These advancements would enhance future epidemiologic studies investigating human exposure to HAHs.

1.5 Toxic Equivalency Factors (TEFs)

Humans are exposed to complex mixtures of environmental pollutants through daily activities. Although HAHs are encountered as mixtures, often only limited toxicity data are available for individual chemicals. This poses a problem when trying to estimate health risks from environmental exposure. Primarily to help prioritize areas of concern for environmental clean-up, various national and international agencies developed toxic equivalency factors (TEFs) for use in risk assessment. A TEF is derived by comparing the potency of a chemical for which limited amount of toxicity data exists, to a chemical for which there is a large body of toxicity (DeVito et al., 1997, Safe,1990). From all the published studies, a chemical is then assigned a point estimate for a TEF value. TEFs can then be used to estimate toxic equivalency (TEQ), which is the sum of weighted potency for each chemical in a mixture.

The TEF approach was originally developed for calculating TEQs in mixtures of PCDDs and PCDFs (Safe, 1990, Birnbaum and DeVito, 1995, DeVito et al., 1997). The actions of these chemicals are mediated by their binding to the Ah receptor (Safe, 1990). Studies demonstrate a rank order correlation between *in vitro* Ah receptor binding affinities and *in vivo* potencies for PCDDs and PCDFs (Bandiera et al., 1985, DeVito et al., 1997). TCDD is considered the prototype chemical because it is the most potent toxicant of this group of chemicals, adequate toxicity data exists, and it binds the Ah receptor with higher affinity than other ligands. The relative potency of these chemicals is influenced not only by the relative binding affinity to the Ah receptor, but also by differences in pharmacokinetic properties (e.g. absorption, distribution, metabolism, excretion) of these chemicals (DeVito et al., 1997). Good correlation has been observed between *in vivo* or *in vitro* responses and TEQ values calculated from the relative concentration of individual congeners in a mixture of PCDFs and PCDDs. TEF values for individual PCDDs and PCDFs are used to estimate the TEQ of TCDD of an environmental mixture, as described below.

E.g., if a mixture contains PCDDs and PCDFs:

$TEQ= \{[PCDDa \times TEF_{PCDDa}] + [PCDDb \times TEF_{PCDDb}]\} + \{[PDCFa \times TEF_{PCDFa}]+[PDCFa \times TEF_{PCDFa}]\} + ...etc.$

$TEQ=$ Summation of $TEF_{congener}$ times the concentration of each individual congener.

A distinction should be made between the terms TEF and relative potency factor (REP). A TEF is an estimate of the potency of a chemical based on all the available experimental data using both *in vivo* and *in vitro* studies (Birnbaum, L., and DeVito, M.J.,1995, Safe, S., 1990). From all the published studies, a chemical is assigned a point estimate for a TEF value. When the potency of a compound relative to TCDD has been obtained in a single *in vivo* or *in vitro* study, it is referred to as a REP value (Van den Berg, et al., 1998). This results in a range of relative potency values for a number of responses. For example, the values calculated in this study for the potency of TCDD-like congeners relative to TCDD are REPs for immunotoxicity. The REPs for PeCDD, 4PeCDF, PCB126 and PCB118 are determined from the ratios of median effective dose (ED_{50}) for suppression of the IgM response to SRBCs of each congener relative to the ED_{50} for TCDD.

Commercial PCB mixtures elicit responses that are similar to those reported for TCDD i.e., thymic atrophy, immunosuppression, carcinogeneisis, hepatotoxicity, porphyria and developmental and reproductive toxicity (Safe, 1984, 1994). A small subset of PCBs, the coplanar PCBs (non-ortho) and *mono*-ortho PCBs, have been shown to bind to the Ah receptor and are thought to contribute largely to the dioxin-like toxicity of these mixtures (Safe, 1984, 1990). The coplanar and *mono*-ortho PCBs competitively displace [^{3}H] TCDD from the cytosolic Ah receptor and these compounds exhibit all of the properties of receptor agonists including the induction of CYP1A1 (Safe, 1992). Quantitative structure activity relationships (SARs) have shown relative Ah receptor binding activities followed the order TCDD> 3,3' 4,4' 5-pentaCB (PCB 126) > 3,3' 4,4'-tetraCB (PCB 77)> 3,3' 4,4' 5,5'-hexaCB (PCB 169) > *mono*-ortho coplanar PCBs (i.e. PCB 118) (Safe, 1992). TEF values for these TCDD-like PCBs have also been proposed (Safe, 1994). This is appropriate, considering that in some environmental mixtures, the overall contribution of PCBs to TEQs exceeds that of

the PCDDs and PCDFs (Kannan et al., 1988).

There are limitations to the TEF approach. For one, it is only useful for Ah-mediated processes. As discussed earlier, there is evidence that TCDD and TCDD-like congeners exert immunotoxic effects through the Ah receptor, but other mechanisms may also be at work. Secondly, the predictive value of TEQs for PCBs, PCDDs and PCDFs may be both species and response specific (Safe, 1992). Evidence is inconclusive at this time as to how susceptible humans are to HAH-mediated immuntoxicity compared to laboratory animals. Also, combined effects of different TCDD-like congeners are assumed to be dose or concentration additive. A number of studies have presented evidence that non-additive effects occur in mixtures similar to those found in the environment. For example, antagonistic effects of mixtures of HAHs on TCDD-induced immunosuppression in the splenic response to SRBC have been documented for 1,3,6,8 TCDF and PCB 153, a common environmental contaminant (Safe, 1990, Smialowicz, et al., 1997). Less potent congeners still have Ah-receptor binding affinities and are effective competitors for the Ah-binding site. However, they do not bind with the necessary affinity to induce Ah-mediated effects. Occupation of the binding site reduces the chance that more toxic dioxin-like compounds will bind to the receptor and decreases the potency of the mixture (Harper et al., 1995). Therefore, in some cases, using the TEF approach may overestimate the immunotoxicity of a mixture. This may be true for environmental mixtures which contain high levels of coplanar PCBs as well as the more toxic PCDDs and PCDFs (Safe, 1994).

1.6 Description of Assays

Immunocompetence is evaluated by quantitating the primary response to SRBCs in both assays examined in this project. As mentioned above, the assays measure different endpoints: the PFC assay counts SRBC specific IgM plasma cells in the spleens of SRBC immunized animals while the SRBC specific IgM ELISA measures total amounts of anti-SRBC IgM in the sera from SRBC immunized animals. Many plasma cells, other than those in the spleen, contribute to the total amount of SRBC specific IgM measured in the sera (Temple et al., 1995).

SRBCs were chosen as the antigen of choice for a variety of reasons. Historically, SRBCs have been the antigen of choice in immunotoxicity screening (Luster et al., 1988). SRBCs are inexpensive, readily available, and are stable for a long time under the proper conditions. In addition, SRBCs are easily adapted for use in the PFC assay and the ELISA. In the PFC assay, lysis of whole SRBCs is readily detectable as plaques formed in semi-solid media. Sheep erythrocytes, as all mammalian RBCs, contain no demonstrable sub-cellular organelles and represent a good supply of plasma membranes. After osmotic lysis, which releases the red cell contents and hemoglobin, the plasma membrane fragments can be purified and adsorbed to wells in a micro titer plate for use in the ELISA assay (Hanahan and Ekholm, 1974). Because the immune response to SRBCs requires T-cells, B-cells and antigen presenting cells (APCs) for the animal to mount an effective humoral response, immunotoxic screening using this antigen evaluates the ability of these cells to act together.

The PFC Assay

The plaque forming cell assay, first described by Jerne and Nordin in 1963, is used to evaluate the humoral response by measuring the number of plasma cells specific to antigen. Plasma cells are mature B cells producing antibodies of one specificity and immunoglobulin class. These B cells are normally restricted to secondary lymphoid organs such as the spleen and lymph nodes. In the standard plaque assay, cells to be tested from an immunized animal are suspended with sheep erythrocytes in a semi-solid medium. Complement is added to the mixture and the entire mixture is transferred to an assay plate and covered with a glass slide. During an incubation period, the test cells secrete antibody. Because the test animals are immunized with whole SRBCs, antibody will be produced to many membrane proteins. The antibody binds to epitopes on the SRBCs in the assay plate and causes lysis of the surrounding SRBCs in the presence of complement. IgM and IgG are the immunoglobulin classes that are the most effective activators of complement (Temple et al., 1995). Cleared areas or plaques, can be visualized in the area surrounding the plasma cell. Counting these plaques equals the number of antigen specific plasma cells per assay plate which can then be converted to the number of plasma cells per specific number of spleen cells and per total spleen.

In our studies, we used a protocol developed earlier in this laboratory for the PFC which measures the response to SRBC with the spleen as a source of test cells. The assay is performed on Day 4 post-immunization for B6C3F1 mice and Sprague-Dawley rats, historically the peak response days for these rodent strains.

THE ELISA ASSAY

The ELISA method was first described by Engvall and Perlman in 1970 as a quantitative assay of immunoglobulin G. In this assay, an enzyme is chemically linked to the antigen or antibody of interest. The unlabeled component (either antigen or antibody) is attached to a solid support, such as the wells of a plastic micro titer plate. The plastic will adsorb a certain amount of any protein. Most commonly, the antigen of interest is attached to the solid support and the binding of antibody in serum samples is assayed. A secondary

enzyme-labeled antibody binds under conditions where non-specific adsorption is blocked. Any unbound material is washed away with buffer solution. Binding is detected by a reaction that converts a colorless substrate into a colored reaction product. Color change can be read in the microtiter plate via spectrophotometer, facilitating data collection (Crowther, 1995).

The SRBC-specific IgM ELISA measures the levels of serum IgM specific to SRBCs (Temple et al., 1993). In the SRBC specific ELISA, SRBC membrane preparations provide the antigen of interest. Animals are immunized with the optimal concentration of SRBCs and at the peak response day, sera are collected. The antigen (SRBC membranes) is applied to microtiter plates, and the binding of serum IgM anti-SRBC is measured via detection with an appropriate secondary antibody and addition of substrate resulting in a characteristic color change. Measurement of the absorbency of this final solution via spectrophotometer at 410 nm allows for quantitation of serum levels of SRBC specific IgM.

It should be noted that the immunization conditions (i.e., concentration of SRBC to inject, kinetics study to determine optimal titer, and production of "internal standard" sera) must be optimized for both assays to detect possible species and strain differences.

2.0 MATERIALS

Animals

Young adult (10 weeks old) female B6C3F1 mice and male Sprague-Dawley rats (sixty days old) were obtained from Charles River laboratory (Raleigh, N.C.) and allowed at least one week to acclimate. Rats were housed 3 per cage and mice 7 per cage containing heat-treated pine shavings (Beta Chips, North Eastern Products, Inc., Warrensburg, NY). Feed (Purina Lab Chow, Ralston Purina Co. St. Louis, MO) and water were provided ad libitum. An ambient temperature of 22°C, relative humidity of 55+\- 5%, and a 12-hr light-dark cycle were provided.

Chemicals and Biochemicals

Sheep red blood cells (SRBCs) in Alsevers' solution and defibrinated SRBCs were obtained from Colorado Serum company, Denver, CO. The same sheep was the source of SRBCs for all experiments. Guinea pig complement, RPMI 1640 media, Earls' balanced salt solution and distilled water, were purchased from GIBCO laboratories.
Phosphate buffered saline (PBS), lauryl sulfate, ethylenediamininetetraactetic acid (EDTA), polyoxyethylenesorbitan monolaurate (Tween 20), trizma hydrochloride, 2,2' azino-bis (3-ethylbenzthiazoline-6-sulfonic Acid) diammonium salt and phosphate-citrate buffer with urea hydrogen peroxide tablets were purchased from Sigma Chemical Company, St. Louis, MO. Affinity purified goat anti-rat IgM-horseradish peroxidase (HRP) (μ) (rat secondary antibody), or affinity purified goat anti-mouse IgM -HRP (μ) (mouse secondary antibody) were purchased from Accurate Chemical and Scientific Corp., Westbury, NY. 2,3,7,8 TCDD was purchased from Radian Corporation and >98% pure as determined by GC-MS. TCDD congeners PeCDD, 4PeCDF, PCB126 and PCB118 were purchased from Accurate Chemical Company.

2.1 METHODS

Development of the antiSRBC IgM specific ELISA:

Isolation of SRBC membranes.

Defibrinated sheep blood diluted 1:1 in Alsever's was centrifuged at 2000x g for 15 minutes at 4°C using a IEC PR-7000 centrifuge with swinging bucket rotor and washed with normal saline. After two washes, the buffy layer (white blood cell (WBC layer) was removed. The cells were then alternately washed with Tris-EDTA and vacuum centrifuged with a fixed angle rotor at 20,000x g for 30 minutes at 4°C until the hemoglobin was removed and the supernatant was clear. Membrane pellets were combined and filtered through 1-2 layers of an ordinary gauze pad to remove the remaining fibrous portion. The combined membranes were placed in dialysis tubing (Spectra/Por ® Membrane MWCO:12-14,000). The suspension was dialyzed for 24 hours at room temperature against 0.1% SDS in PBS. The protein concentration of the dialyzed membrane solution was measured using a BCA Protein Assay Reagent Kit (Bio-Rad). SRBC membrane preparations were stored at -20° C at a protein concentration of @ 1 mg/ml.

SRBC IgM ELISA.

For performance of the ELISA, stored SRBC membrane preparation was allowed to thaw and diluted to 2.0 μg/ml in PBS. Each well of 96-well Immulon-2 ELISA microtiter plates (flat-bottom, Dynatech Labs) was coated with 125μl of this solution using a Costar Multichannel Pipettor. The plates were incubated at 4°C for no less than 16 hrs. On the day of the assay, plates were hand decanted and washed three times with 0.05% Tween/PBS using a Nunc-Immuno Wash 12 plate washer. Plates were blotted and blocked with 4% nonfat milk solution in distilled water for one hour at 37°C. Plates were washed as described above. Wells in the second column were coated with 200μl of a 1:8 dilution of test serum or internal control serum as appropriate. Serum samples were sequentially diluted from 1:8-

1:4096 in PBS. The last wells of each row contained only PBS and provided the negative control. Plates were incubated for 1 hour at 37°C and then washed as described above. The appropriate secondary antibody (e.g. affinity purified goat anti-rat IgM-HRP (μ), or affinity purified goat anti-mouse IgM -HRP (μ)) was diluted in 4 % nonfat milk solution and 100μl was added per well. The optimal concentration of secondary antibody was usually 1:2500 (mouse) and 1:2000 (rat) with only slight variability due to the different lots of antibody. The plate was incubated for 1 hour at 37°C, then washed as described earlier. Lastly, 100μl of Peroxidase substrate was pipetted per well and the plate was incubated for 45 minutes. The absorbency of this final solution was measured via spectrophotometer (SpectraMax 250 plate reader) at 410 nm.

ELISA data analysis.

IgM levels could not be expressed in protein concentration or activity since a standard IgM specific to SRBC membranes was not available. Studies were evaluated using the linear region of a log/log plot of 8 serial twofold dilutions. In order to make comparisons with and between studies, an absorbance was chosen which fell within the data points of the study (0.5 OD) and the titer of the serum needed to produce that absorbance was extrapolated from the equation resulting from the linear portion of the curve. Softmax software (Molecular Devices) was used for this analysis.

NOTE: For more detail see the complete SRBC Membrane and SRBC ELISA protocols in the appendix.

SRBC/PFC Assay

Four days following immunization, animals were bled for serum to be used for SRBC IgM ELISA prior to removal of the spleen, thymus and liver, which were weighed. Test animals were killed by CO_2 anoxia followed by cervical dislocation. Spleens were removed aseptically and placed in a tube containing RPMI-1640 media supplemented with 5% FCS and 0.1% gentamiacin. The contents of each tube were transferred to a sterile Stomacher 80 bag. Spleen cells were disbursed using a Stomacher Lab-Blender (80) (Tekmar, Cincinnati, OH). Cells were removed from the bag and transferred back into the original tube. The bag was rinsed with RPMI and this was added to the tube.

The single cell suspension was allowed to sit on ice for 10 minutes. Contents were carefully pipetted into a separate tube, leaving debris in the original tube. Dilutions of the suspension were made in media at 1:40 and 1:80 (mice) and 1:50 and 1:100 (rats). Cell counts were taken on a Coulter Counter Model ZBI (Coulter Electronic Inc., Hialeah, Fl.) and viabilities determined by using the trypan blue exclusion method. SRBC (Colorado Serum) were washed three times with saline and resuspended to 50% with RPMI media. Reconstituted guinea pig complement previously adsorbed with one drop of washed SRBC and frozen, was thawed, diluted 1:3 with assay media and held on ice. Agar was prepared using 0.5% Bacto-Agar (Difco, Detroit, MI) and 0.05 % diethyl aminoethyl cellulose (DEAE-dextran, Pharmacia Fine Chemicals, Uppsala, Sweden) in Earles' balanced salt solution (EBS). Agar solution was maintained at 47°C in a constant temperature water bath.

Spleen cells, SRBC, agar, and complement were added to 10 x 75 mm glass tubes in duplicate. Solutions were vortexed briefly, poured into 100 mm petri plates (Costar, Cambridge, MA) and covered with 45 x 50 mm glass cover slips (Erie Scientific, Portsmouth, NH). Plates were incubated at 37°C and 5% CO_2 for three hours. After incubation, plaques were counted.

Note: see the complete PFC/SRBC protocol in the appendix for more detail.

Determination of peak day(s) of anti-SRBC IgM response as measured by ELISA and SRBC/PFC for rats and mice:

Optimal time of PFC/SRBC and anti-SRBC ELISA response following immunization to SRBC from Colorado Serum in female B6C3F1 mice.

Twenty-eight (28) female B6C3F1 mice (10 weeks old on the day of the experiment) were divided into four (4) groups with seven (7) mice per group. All mice were immunized by intravenous (iv) injection with 0.2 ml of an optimal concentration (i.e., 0.25×10^8 SRBC/mouse or 1.25×10^8 SRBC/ml) of SRBC. Mice were immunized 6, 5, 4, and 3 days prior to the day of the SRBC/PFC. On the day of the PFC assay, mice were bled by jugular transection under CO_2 anoxia and blood was collected in small serum separation tubes. Sera were collected and placed in small, labeled microfuge tubes at 75 μl/ tube, with the top of the tube wrapped in parafilm and stored in the freezer until the day of the ELISA.

Optimal time of PFC/SRBC and antiSRBC ELISA response following immunization to SRBC from Colorado Serum in male Sprague-Dawley rats.

Thirty (30) male Sprague-Dawley rats (60 days-old at the time of the experiment) were divided into five (5) groups with 6 rats/ group. All rats were immunized by iv injection with 0.5 ml of a 2.0 % suspension of SRBC (i.e 4x10 8 SRBC/ml or 2x10 8 SRBC/rat). Rats were immunized 7, 6, 5, 4, or 3 days prior to the day of the SRBC/PFC. On the day of the PFC assay, rats were bled for sera used in the ELISA assay. Rats were anesthetized using Metofane and exsanguinated via the abdominal aorta. Blood was placed in serum separation tubes and after 30-45 minutes at room temperature, centrifuged for 7 minutes at 2500 rpm. Sera were collected and placed in small labeled microfuge tubes at 200μl/tube, with the top of the tube wrapped with Parafilm®, and stored in the freezer.

Measurement of SRBC response by PFC and ELISA following administration of TCDD or TCDD congeners in mice.

Preliminary study to predict immunosuppressive doses of TCDD or TCDD congeners in mice.

A preliminary study was conducted in order to determine appropriate chemical concentrations of TCDD or TCDD-like congeners (PeCDD, 4PeCDF, PCB126 and PCB118) prior to PFC/ELISA comparison studies. Doses that were anticipated to be immunosuppressive were selected for all chemicals.

Comparison studies examining immunosuppression by TCDD and TCDD-congeners.

The effect of a single oral dose with TCDD or TCDD-like congeners (PeCDD, 4PeCDF, PCB126 and PCB118) on the SRBC/PFC and antiSRBC ELISA response in female B6C3F1 mice was determined in a series of experiments. For TCDD, and each TCDD-like congener, 6 groups of 14 female B6C3F1 mice/group (8 weeks old on day of receipt) were weighed and dosed orally with corn oil or the test chemical in corn oil in a volume of 10 ml/kg.

Dosing solutions of TCDD and TCDD-like congeners were prepared from stock solutions containing 1 mg/kg of chemical in 10 ml of corn oil. The stock solutions were

prepared by dissolving TCDD or TCDD-like congeners in acetone, mixing the acetone solution with corn oil, and then removing the acetone by evaporation (DeVito, M.J., et al., 1993). Mice were weighed and dosed once via gavage with corn oil, TCDD, or TCDD-like congeners in corn oil in a volume of 10 ml/kg.

One week later (7 days), all mice were immunized with an i.v. injection of an optimal concentration (i.e., 0.25×10^8 SRBC/mouse or 1.25×10^8 SRBC/ml) of SRBC. The SRBC/PFC assay was performed using seven (7) mice/group four days after immunization. Serum was collected from the remaining seven (7) mice/group five days after immunization (i.e. the day of peak IgM response) and stored in the freezer until analysis by ELISA.

Statistics and Statistical models.

All data were analyzed using Dunnett's multiple comparison t-test (Dunnett, 1955) with a $p < 0.05$ considered significant.

A statistical model, provided by Mike DeVito using SigmaStat, uses a shaping curve to fit the raw data and generated the "ED_{50}" values from the PFC and ELISA data for TCDD and TCDD-like congeners. The model has been used to describe the dose-response relationship for several different responses in animals treated with TCDD, including immunological responses (DeVito et al., 1994, 1997, McGrath et al., 1995). The shaping function, an adaptation of the Hill equation, is given as :

$$y = E_0 - [(E_{max} \times X^n)/ (b^n + X^n)]$$

where X is the dose, E_0 is the estimated background when X is 0, E_{max} is the estimated maximun possible decrease below background in response, b is the estimated ED_{50} and n is a shaping parameter.

The biologically important characteristics of this function are that it has lower and upper extremes that are determined by the biological response alone, independent of dose. There is a natural background or level of the immune response associated with the lower extreme. This is the response of the immune system to challenge with SRBC observed with the control group. The upper asymptote is associated with saturation of the response: continuing to increase the dose of the compound will not suppress the immune response to

SRBC beyond a certain point.

(See DeVito et al., 1997 for further details.)

3.0 RESULTS

Time course of SRBC-specific IgM in mice and rats.

Both mice and rats immunized with SRBC showed a peak PFC response in spleens on Day 4 post-immunization (Figure 1 A, Figure 2 A). A similar time course measuring serum IgM- specific antibody showed a peak at Day 5 in B6C3F1 female mice and a peak on Day 6 in male Sprague-Dawley rats (Figure 1 B, Figure 2B).

Preliminary study: determining an immunosuppressive dose of TCDD and TCDD congeners.

In Table 1, results from the preliminary study demonstrated significant suppression of the SRBC-specific PFC response and SRBC-specific IgM titers at 0.5 μg TCDD/kg. 1000 μg PCB 126 /kg, 20 μg PeCDD/kg, and 10 μg 4PeCDF/kg. A dose of 65 mg/kg of PCB 118 significantly increased the SRBC-specific PFC response.

There were no significant differences in body weights among treatment groups. Organ weight effects were observed, as expected, and are expressed as organ weight to body weight ratios in Table 2. Statistically significant decreases in spleen weight to body weight and thymus weight to body weight ratios were observed with 0.5 μg TCDD /kg and 1000 μg PCB126/kg. Significant increased in liver weight to body weight ratios were observed with 0.5 μg TCDD /kg, 1000 μg PCB126/kg, 20 μg PeCDD/kg, and 65 mg PCB118/kg. Spleen weight to body weight ratio was increased with 65 mg PCB118/kg. The results of this study, in combination with reported TEF values, were used to determine appropriate doses of these HAHs for subsequent experiments.

Suppression of the PFC and SRBC-IgM response by the administration of TCDD and TCDD-like congeners.

Effects on Body and Organ Weights.

A single dose of TCDD or TCDD-like congeners did not produce any treatment-related mortality. Body weight changes and spleen, thymus and liver weights were examined on the day of the PFC assay for all chemicals tested. Body weights at the time of sacrifice were unaffected by treatment with any chemical. Organ weight: body weight ratios are shown in Tables 3-7.

As seen in Table 3, a significant decrease in spleen to body weight was observed at the highest dose of TCDD (3.0 μg/kg). A significant decrease in thymus to body weight as compared to controls was observed at 1.0 and 3.0 μg TCDD/kg. Liver to body weight ratios were significantly increased, in a dose-related manner, at all doses of TCDD relative to controls.

In Table 4, only the liver to body weight ratios were significantly affected at three highest doses of PCB 126 (100 μg/kg, 300 μg/kg and 1000 μg/kg). Administration of the highest doses of PeCDD (4.0 μg/kg and 12.0 μg/kg, Table 5) resulted in a significant decrease of spleen to body weight ratios, while only the highest dose significantly increased liver to body weight ratios relative to controls.

For 4PeCDF-treated mice, significant decreases were observed in spleen to body weight ratios (90 μg/kg dose) and thymus to body weight ratios (all doses, Table 6). This decrease in thymus to body weight ratio is apparently due to the fact that the thymus weights in this experiment control group had larger thymus weights compared to all other control groups. However, the thymus to body weight ratios for the dose groups are comparable to those of all other control groups.

A significant increase in liver to body weight ratio was observed at the highest dose of 4PeCDF as compared to controls.

Only the highest doses of PCB 118 (120 mg/kg, 240 mg/kg and 420 mg/kg) increased liver to body weight ratios relative to controls (Table 7).

Suppression of the PFC and SRBC-IgM response by the administration of TCDD and

TCDD-like congeners.

Plots of the treatment doses versus data generated from both assays demonstrated good dose-response curves for all chemicals tested (Figures 3-7). Following treatment with TCDD, mice showed significantly immunosuppression with 0.5, 1.0 or 3.0 μg/kg doses as measured by PFC (Figure 3A) and with 0.3, 0.5. 1.0 or 3.0 μg/kg doses as measured by serum IgM (Figure 3B). In mice treated with PCB 126, both assays measured significant depression at all doses (Figure 4A, B). Treatment with 4PeCDF resulted in immunosuppression of the PFC response at 15.0 μg/kg, 30 μg/kg, or 90 μg/kg (Figure 5A) while serum IgM titers were significantly depressed at all doses (Figure 5B). Administration of 1.2, 2.0, 4.0 or 12.0 μg/kg PeCDD significantly depressed the PFC response, while serum IgM levels were significantly depressed at all treatment doses (Figure 6A, B). Only the highest dose of PCB 118 (480 mg/kg) caused significant suppression of the PFC response, while 120, 240 and 480 mg/kg PCB 118 significantly depressed serum IgM levels.

Calculated "ED_{50}"and REP values.

The "ED_{50}", or the dose needed to suppress 50% of the immune response as compared to controls, is reported for each chemical in Table 8. For all chemicals except PCB 126, the "ED_{50}" values generated by the model using the ELISA results were approximately one-half those predicted by the PFC data.

Relative potency values were calculated using the "ED_{50}" values from Table 8 and are presented in Table 9. There was good agreement between the PFC and ELISA generated REPs. For comparison, REP values for CYP1A1 enzyme induction from Birnbaum and DeVito (1995) REP ranges from Safe (1990) and the World Health Organization (WHO) (adapted from Van Der Berg, 1998) appear to the left of the PFC and ELISA REPs in Table 9. With the exception of PCB 126, the rank order of the ED_{50}s and immunotoxic potency agrees with the rank order of Ah receptor activity and WHO TEF values for TCDD and TCDD-like congeners. The exception can be explained by examining the dose-response curves for PCB 126 (Figures 4A and B) which dropped sharply due to the dose selection. As a result, the data poorly fit the model used to generate ED_{50} values, because, as described in the methods section, the model is based on an equation giving an asymptotic curve which has

characteristic plateaus at very high and very low doses.

In general, the SRBC ELISA data fit the statistical model better than the PFC due to the lower variability of the ELISA assay. SRBC ELISA and PFC immunotoxicity REP values for PeCDD and 4PeCDF are closer to REP values reported by DeVito (1997) for CYP1A1 induction, while those for PCB 126 and PCB 118 agree more closely with WHO TEF values.

4.0 DISCUSSION

The immune system is one of the most sensitive targets for TCDD toxicity in experimental animals. Alteration of immune endpoints by TCDD (e.g. altered lymphocyte subsets, increased susceptibility to viral and bacterial infection) has been reported at body burdens in animal models that are equivalent to human background exposures to TCDD and dioxin-like chemicals (DeVito, et al., 1995). *In vitro* studies using mouse and human immune cells have demonstrated that similar concentrations of TCDD produce identical effects. For example, the LOEL for inhibition of proliferation of mouse and human thymocytes was 0.1 nM of TCDD (Greenlee et al., 1975). LOELs for inhibition of IgM secretion and proliferation for mouse and human lymphocytes were 3.0 nM TCDD and 0.3 nM TCDD, respectively (Wood et al., 1993). However, evidence for dioxin-mediated immunotoxicity in human subjects is inconclusive, as discussed earlier. Some epidemiological studies have documented subtle immune alterations in populations heavily exposed to dioxins, but the clinical importance of these findings is unknown.

In this study, 0.3 μg TCDD/kg in female B6C3F1 mice was sufficient to result in immunosuppression as detected by SRBC ELISA. The corresponding body burden in mice treated with this intial dose 7 days later (the day of challenge with SRBC antigen) is 112 ng TCDD/ kg (assuming a half-life of 10 days in the mouse, and 80% absorption of the initial dose). The average body burden of TCDD in the general population is estimated to be 1.1 ng TCDD/ kg body weight or 13 ng TEQ/kg body weight for total PCDDS, PCDFs, and PCBs (DeVito et al., 1995). Therefore, there is approximately a ten-fold difference between the tissue concentration necessary to cause immunosuppression in mice and the background levels of human exposure. A margin of exposure (MOE) of 100 is used by the U.S. EPA in risk assessment methods. Humans also receive low daily doses of dioxinlike chemicals through dietary exposure. Human daily exposure to TCDD or dioxin-like chemicals has been

estimated as 3-6 pg/TCDD TEQs/ day in the United States (Schecter et al., 1994).

Studies to date with various immunotoxic chemicals have proven the SRBC-specific serum IgM ELISA to be at least as sensitive as the spleen IgM-PFC assay. In studies by Temple et al (1993, 1995), B6C3F1 female mice were dosed with the immunosuppressive drugs benzo(a)pyrene (B[a]P), morphine, and cyclophosphamide. Administration of these chemicals produced identical lowest observed effect levels (LOELs) when using the PFC assay or SRBC ELISA, leading the investigators to conclude that the SRBC-specific serum IgM ELISA was as sensitive as the spleen IgM-PFC assay in measuring effects on the humoral immune system.

We have shown that the SRBC-ELISA, performed on the peak day of IgM production, is a more sensitive assay for detecting immunosuppression of the SRBC response by TCDD and TCDD-like congeners in the B6C3F1 mouse. For four out of five chemicals tested, the ED_{50} for suppression of the immune response to SRBC is approximately halved when using the SRBC specific IgM assay as compared to the SRBC PFC. LOELs for immunosuppression were significantly decreased when measuring serum SRBC specific IgM titers as compared to splenic SRBC specific PFCs (Table 10), with the exception of PCB 126.

The dosing range for PCB 126 was not ideal for these comparison studies, as lower doses should have been included in the PCB 126 studies.

Time course studies by Temple (1993) and demonstrated herein revealed that IgM peaks in the serum 1 day later than IgM antibody-forming cells in the spleen in female B6C3F1 mice. However, Temple (1993, 1995) used sera collected on the day of the PFC assay (Day 4 post-immunization). We compared SRBC ELISA results using Day 4 and Day 5 serum.

As can be seen in Table 10, both SRBC ELISA assays were more sensitive to immunotoxicity than the PFC assay, regardless of the day of serum collection, with the exception of PCB 126. However, LOELs for the SRBC ELISA performed using sera from the peak day of SRBC IgM production were lower for PCB 118 and PeCDD than using day 4 serum. This emphasizes the importance of testing peak day sera using the SRBC membrane ELISA for maximum sensitivity of the assay.

Serum IgM may represent a more physiologically relevant parameter for immuntoxicity testing. The IgM molecule has a half-life of 5.1 days in the body (Waldman et al., 1970). Serum IgM is a pool of accumulated IgM produced by the spleen, bone marrow and other lymphoid organs. SRBC-specific IgM levels in the sera reflect an aggregate immune response in the entire organism, while the PFC assay reports the number of splenic antibody-producing cells at a specific point in time.

The PFC assay also introduces more variability, as can be observed in the comparatively large SEM values for all PFC studies reported here. This is probably due to several factors, including the number of steps and manipulations of the cells employed in the assay, which necessitate a minimum of two to four technicians for successful and timely completion of the assay. By contrast, the SRBC ELISA can be completed by one technician in four hours and is a relatively simple procedure. Another distinct advantage of the ELISA is that the serum IgM can be measured in small blood samples, allowing multiple studies to be done on the same animals. Samples can be frozen, and analyzed at another time or by another laboratory. Alternatively, there is the potential for using automated robotic ELISA systems, making this assay ideal for screening numerous compounds.

TABLE 1: Preliminary Study Results.
Doses were chosen that were predicted to be immunosuppressive for TCDD and TCDD-like congeners.

Chemical	DOSE	Mean PFC/E6 Cells ± SEM	Mean Titer to give 0.5 OD ± SEM
None (Corn Oil)	0	1456.833± 128.394	477.143± 36.641
TCDD	0.5 μg/kg	670.571± 125.826*	135.667± 11.382*
PCB 126	1000 μg/kg	31.286± 7.063*	105.571± 10.989*
PeCDD	20 μg/kg	745.000± 85.631*	225.143± 23.760*
4PeCDF	10 μg/kg	891.714± 88.513*	272.833± 44.639*
PCB 118	65 mg/kg	2106.286± 169.180*	500.714± 34.902

* p≤ 0.05, Dunnett's t-test. n=7

TABLE 2: Ratio of Organ: Body Weight from preliminary study.

Chemical	DOSE	SP:BW±SEM	TH:BW±SEM	LV:BW±SEM
None (Corn Oil)	0	4.486± 0.116	2.346± 0.109	47.620± 0.572
TCDD	0.5 μg/kg	3.652± 0.116*	1.859± 0.091*	55.989± 1.147*
PCB 126	1000 μg/kg	2.597± 0.095*	0.505± 0.099*	79.330± 1.693*
PeCDD	20 μg/kg	4.442± 0.264	2.115± 0.077	59.645± 0.866*
4PeCDF	10 μg/kg	4.301± 0.065	2.168± 0.086	50.763±0.983
PCB 118	65 mg/kg	5.472± 0.128*	2.476± 0.092	51.933± 0.983*

* p≤ 0.05, Dunnett's t-test. n=7
SP= spleen weight, TH= thymus weight, LV= liver weight, BW=body weight
No change in body weight was detected between controls and treatment groups.

TABLE 3: Ratio of Organ: Body Weights for TCDD-dosed mice.

Dose	SP:BW	TH:BW	LV:BW
None (Corn oil)	4.411 ±0.192	2.518 ±0.085	46.308 ±0.490
0.1 ug/kg TCDD	4.435 ±0.184	2.399 ±0.078	49.993 ±0.834*
0.3 ug/kg TCDD	4.276 ±0.202	2.495 ±0.104	51.853 ±1.261*
0.5 ug/kg TCDD	3.993 ±0.100	2.275 ±0.110	52.408 ±1.253*
1.0 ug/kg TCDD	4.022 ±0.160	2.107 ±0.057*	53.016 ±0.780*
3.0 ug/kg TCDD	3.746 ±0.137*	1.928 ±0.152*	55.463 ±1.206*

* $p < 0.05$, Dunnett's t-test. n=7
SP=spleen weight, TH=thymus weight, LV=liver weight, BW=body weight.
No change in body weight was detected between controls and treatment groups.

TABLE 4: Ratio of Organ: Body Weights for PCB 126-dosed mice.

Dose	SP:BW	TH:BW	LV:BW
None (Corn oil)	4.472 ±0.155	2.635 ±0.138	46.969 ±1.368
10 μg/kg PCB 126	4.832 ±0.269	2.356 ±0.138	48.135 ±1.393
50 μg/kg PCB 126	4.460 ±0.206	2.330 ±0.190	50.459 ±1.402
100 μg/kg PCB 126	4.726 ±0.225	2.536 ±0.133	54.968 ±0.860*
300 μg/kg PCB 126	3.914 ±0.077	2.529 ±0.144	52.872 ±1.154*
1000 μg/kg PCB 126	4.265 ±0.241	2.487 ±0.332	57.049 ±1.690*

* $p < 0.05$, Dunnett's t-test. n=7
, SP=spleen weight, TH=thymus weight, LV=liver weight, BW=body weight.
No change in body weight was detected between controls and treatment groups.

TABLE 5: Ratio of Organ: Body Weights for PeCDD-dosed mice.

Dose	SP:BW	TH:BW	LV:BW
None (Corn oil)	4.831 ±0.227	2.431 ±0.086	49.903 ±1.529
0.4 μg/kg PeCDD	4.717 ±0.101	2.670 ±0.096	48.584 ±0.850
1.2 μg/kg PeCDD	4.607 ±0.193	2.525 ±0.120	50.363 ±0.862
2.0 μg/kg PeCDD	4.603 ±0.170	2.589 ±0.106	50.392 ±3.090
4.0 μg/kg PeCDD	4.094 ±0.142*	2.491 ±0.097	51.476 ±1.963
12.0 μg/kg PeCDD	3.856 ±0.130*	2.341 ±0.071	53.398 ±1.000*

* $p < 0.05$, Dunnett's t-test. n=7
SP=spleen weight, TH=thymus weight, LV=liver weight, BW=body weight.
No change in body weight was detected between controls and treatment groups.

TABLE 6: Ratio of Organ: Body Weights for 4PeCDF-dosed mice.

Dose	SP:BW	TH:BW	LV:BW
None (Corn oil)	4.663 ±0.141	2.942 ±0.115	48.079 ±0.851
3.0 μg/kg 4PeCDF	4.816 ±0.164	2.584 ±0.071*	49.656 ±0.825
9.0 μg/kg 4PeCDF	4.501 ±0.126	2.538 ±0.101*	49.566 ±0.467
15 μg/kg 4PeCDF	4.406 ±0.113	2.490 ±0.101*	51.692 ±.889*
30 μg/kg 4PeCDF	4.298 ±0.093	2.563 ±0.083*	52.546 ±0.591*
90 μg/kg 4PeCDF	3.814 ±0.104*	2.445 ±0.060*	53.879 ±1.000*

* $p < 0.05$, Dunnett's t-test. n=7
SP=spleen weight, TH=thymus weight, LV=liver weight, BW=body weight.
No change in body weight was detected between controls and treatment groups.

TABLE 7: Ratio of Organ: Body Weights for PCB 118-dosed mice.

Dose	SP:BW	TH:BW	LV:BW
None (Corn oil)	4.659 ±0.156	2.696 ±0.094	45.960 ±1.083
15 mg/kg PCB118	4.823 ±0.259	2.669 ±0.133	47.452 ±1.018
30 mg/kg PCB118	4.736 ±0.297	2.669 ±0.162	46.223 ±1.218
60 mg/kg PCB118	4.492 ±0.144	2.458 ±0.067	47.966 ±0.759
120 mg/kg PCB118	4.685 ±0.170	2.672 ±0.083	51.019 ±0.921*
240 mg/kg PCB118	5.116 ±0.207	2.744 ±0.061	57.150 ±1.818*
480 mg/kg PCB118	4.374 ±0.201	2.794 ±0.076	66.158 ± 3.413*

* $p < 0.05$, Dunnett's t-test. n=6
SP=spleen weight, TH=thymus weight, LV=liver weight, BW=body weight.
No change in body weight was detected between controls and treatment groups.

TABLE 8: "ED 50" Values.

Chemical	PFC Data			ELISA Data		
	"ED 50"	SE	p value	"ED 50"	SE	p value
TCDD	0.715	0.262	0.072	0.307	0.042	0.006
PeCDD	1.269	0.211	0.009	0.852	0.157	0.012
PCB 126	15.185	15.940	0.411	2.413	1.447	0.194
4PeCDF	21.734	8.140	0.076	10.119	2.081	0.017
PCB 118	306.401	137.637	0.090	158.856	64.818	0.072

TABLE 9: TEF, REP Values.

Chemical	WHO TEFs*	REP Values**	REP ranges #	Rank	PFC REP	ELISA REP	Rank
TCDD	1	1	1	1	1	1	1
PeCDD	1.000	0.500	0.590-0.053	2	0.563	0.360	2
4PeCDF	0.500	0.140	0.800-0.120	3	0.033	0.030	4
PCB 126	0.100	0.010	0.300-0.006	4	0.047	0.127	3
PCB 118	0.0001	0.000001	0.00045-0.0000014	5	0.0023	0.0019	5

* from Van Der Berg, 1998.
** from Birnbaum and DeVito, 1995.
# from Safe, 1990, 1992.

TABLE 10: Comparison of PFC, SRBC ELISA (Day 5) and SRBC ELISA (Day 4) LOELS.

Chemical	PFC LOEL	SRBC ELISA LOEL	SRBC ELISA (Day 4) LOEL
TCDD	0.5 μg/kg	0.3 μg/kg	0.3 μg/kg
PCB126	10 μg/kg	10 μg/kg	10 μg/kg
PeCDD	1.2 μg/kg	0.4μg/kg	1.2 μg/kg
4PeCDF	15 μg/kg	3μg/kg	3 μg/kg
PCB 118	480 μg/kg	120 μg/kg	240 μg/kg

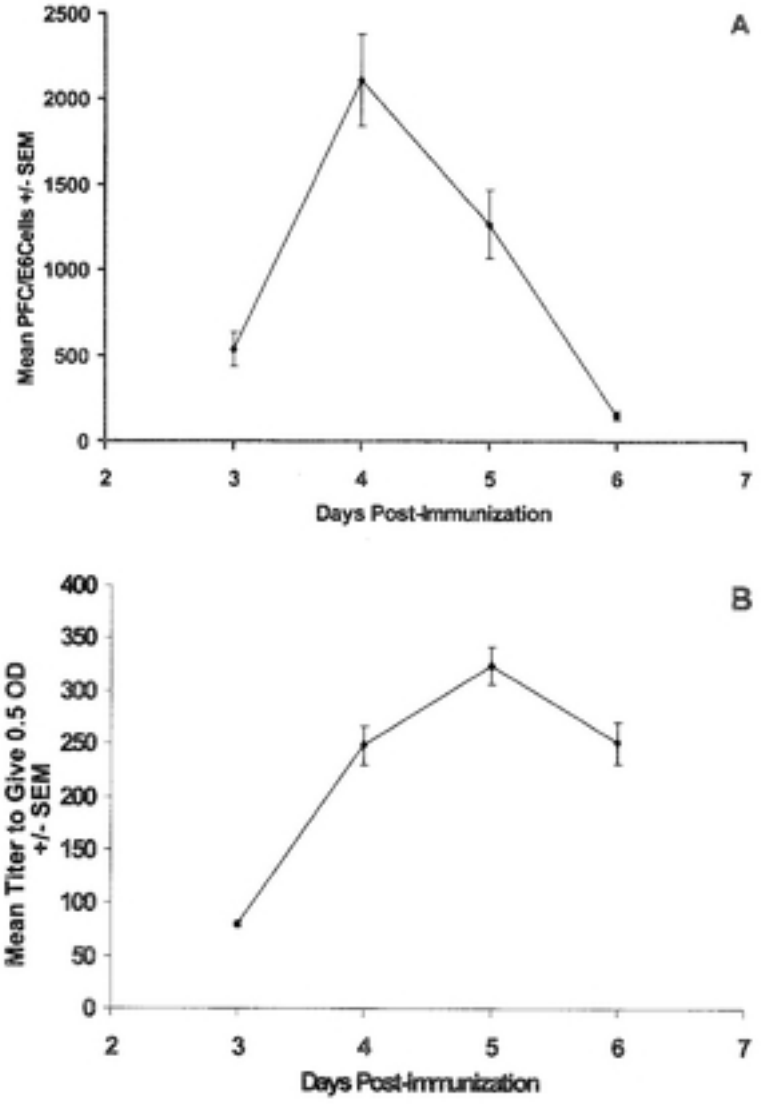


Figure 1. Day Post-Immunization of optimal response of PFC (A) and serum anti-SRBC IgM (B) in B6C3F1 female mice. N=7

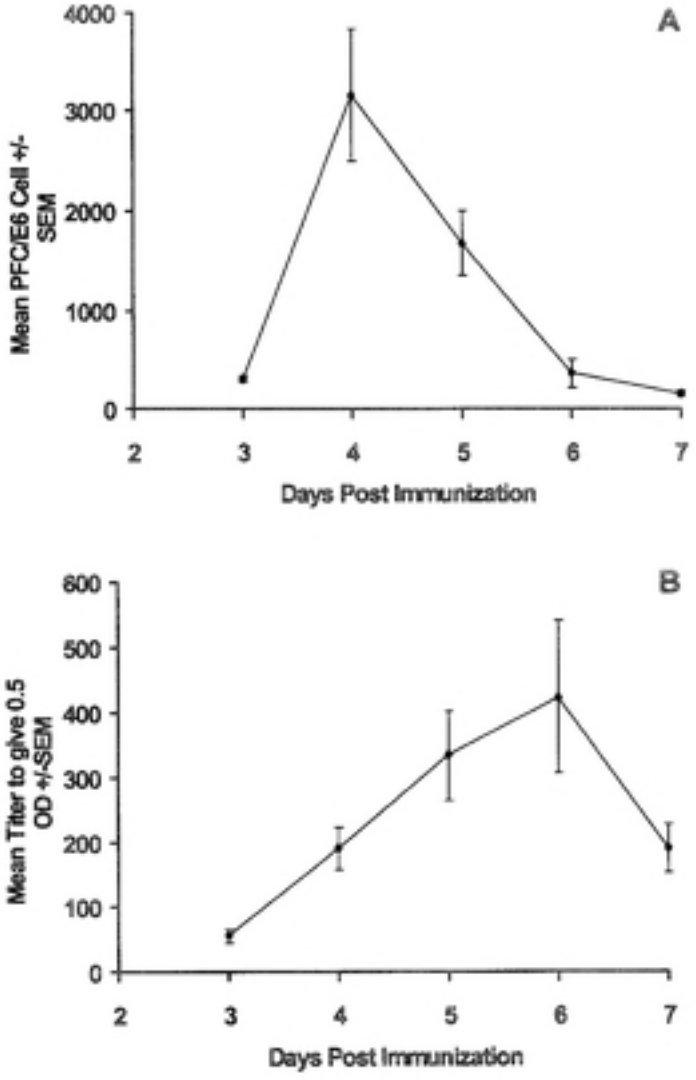


Figure 2. Day Post-immunization of optimal response of PFC (A) and serum anti-SRBC IgM (B) in Sprague-Dawley male rats. N=6

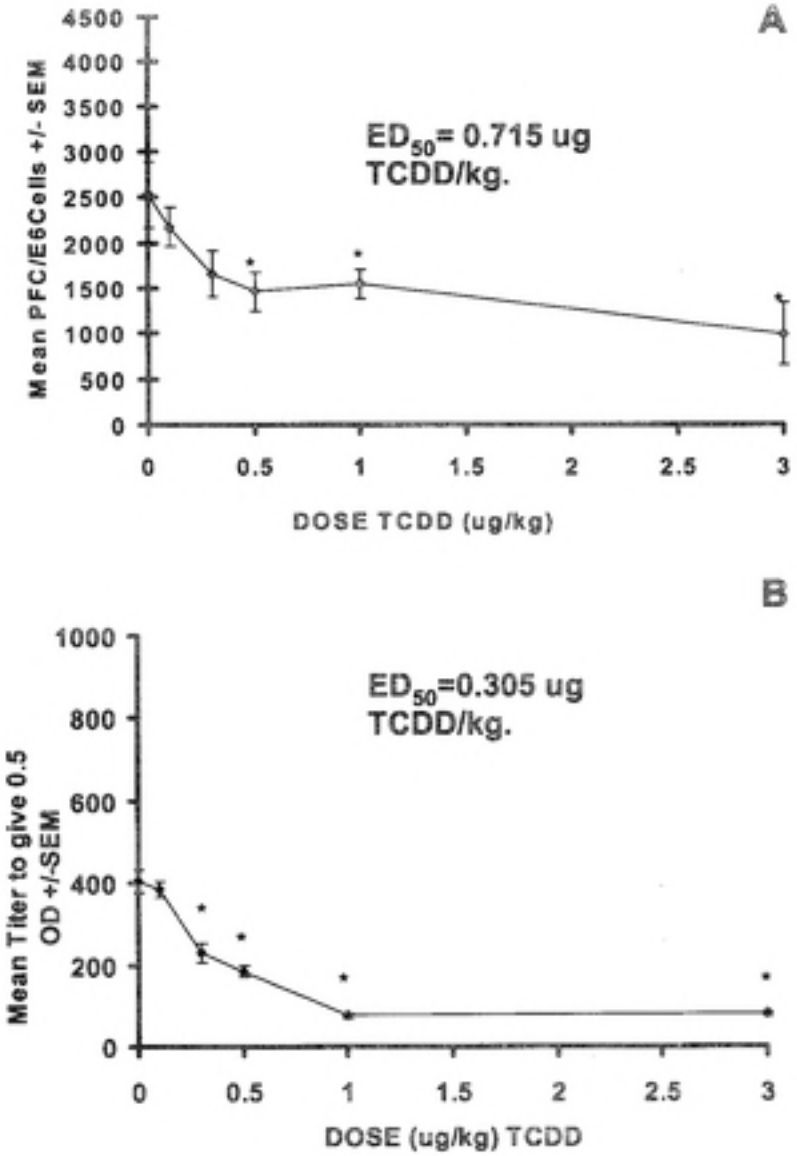


Figure 3. SRBC PFC assay(A) and antiSRBC IgM ELISA (B) using TCDD as the test chemical in B6C3F1 female mice. N=7

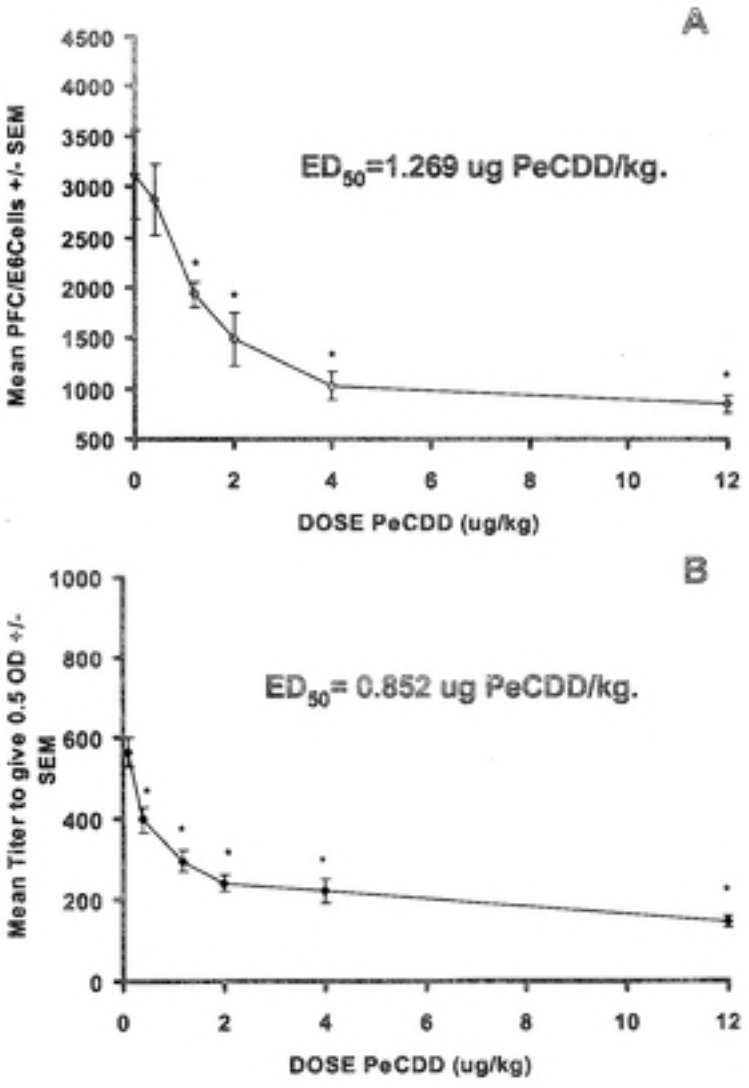


Figure 4. SRBC PFC assay (A) and AntiSRBC IgM ELISA (B) using PeCDD as the test chemical in B6C3F1 female mice. N=7

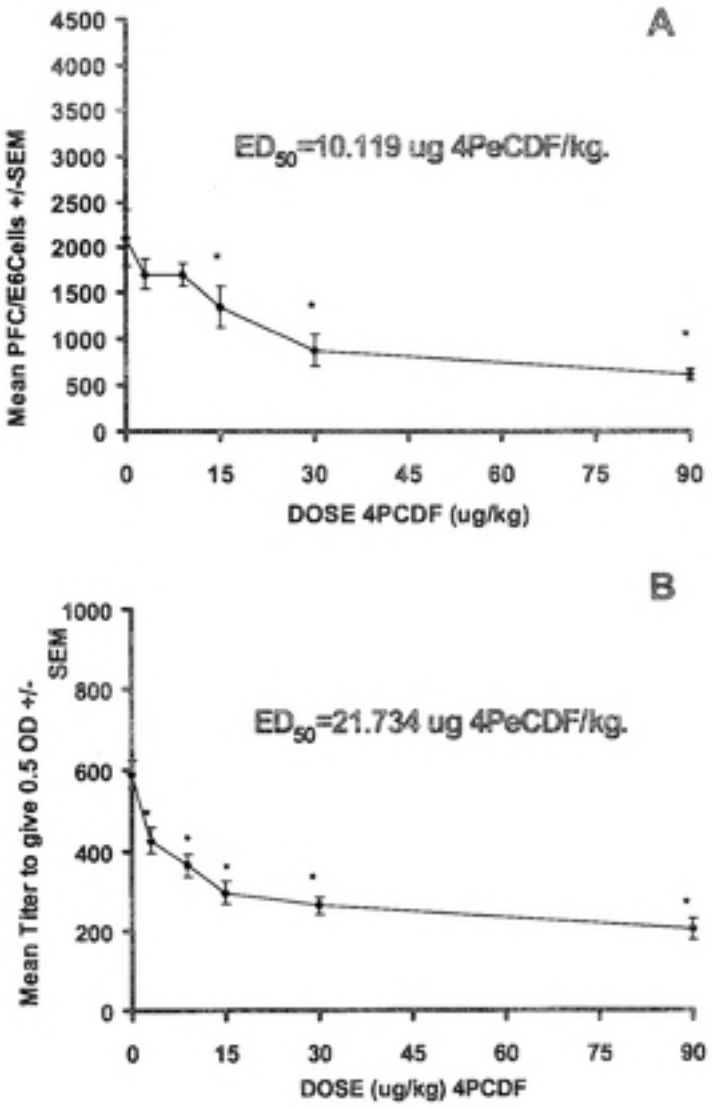


Figure 5. SRBC PFC assay (A) and AntiSRBC IgM ELISA (B) using 4PeCDF as the test chemical in B6C3F1 female mice. N=7

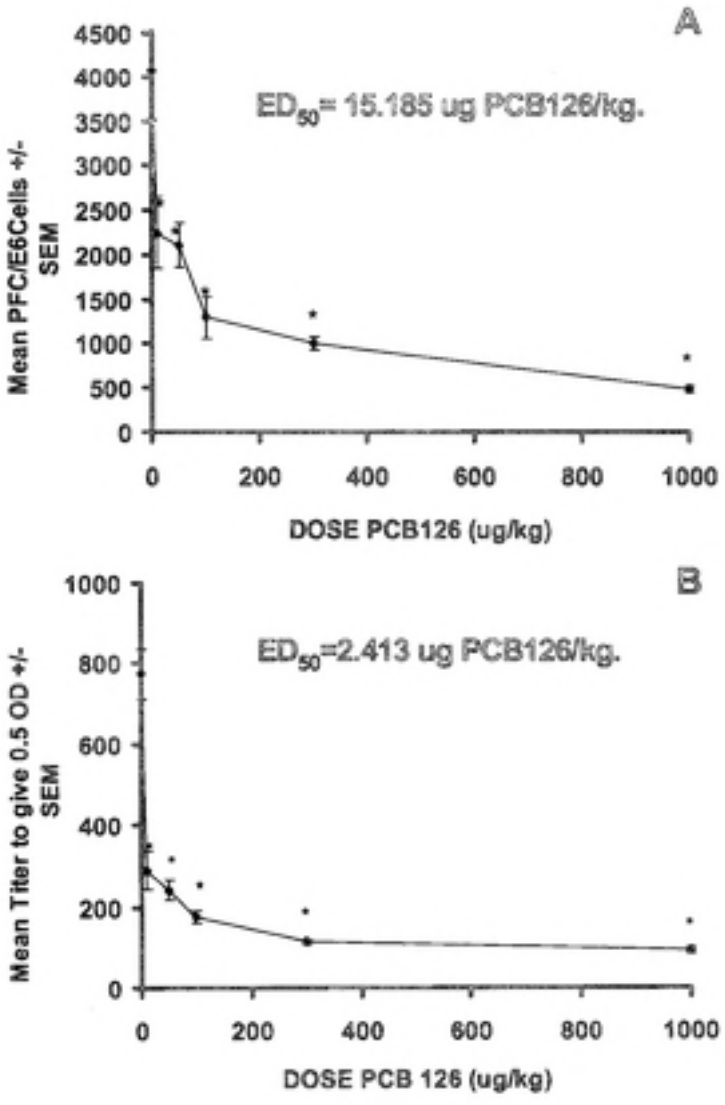


Figure 6. SRBC PFC assay (A) and Anti-SRBC IgM ELISA (B) using PCB126 as the test chemical in B6C3F1 female mice. N=7

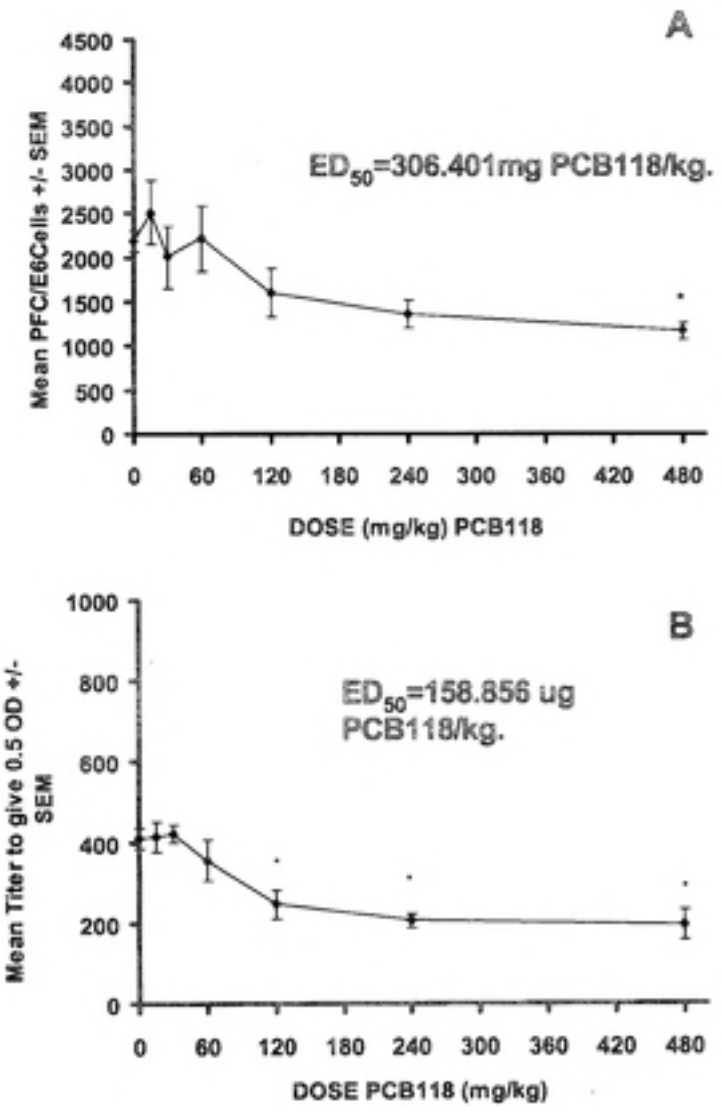


Figure 7. SRBC PFC assay (A) and AntiSRBC IgM ELISA (B) using PCB118 as the test chemical in B6C3F1 female mice. N=7

REFERENCES

Benner, R., Hiijams, W., Haaijman, J. (1981). The bone marrow : the major source of serum immunoglobulibns, but still a neglected site of antibody formation. Clinical and Experimental Immunology: 46(1): 1-8.

Birnbaum, L.S. (1995) Workshop on Perinatal Exposure to Dioxin-like compounds. V. Immunological Effects. Environmental Health Perspectives: 103. Suppl. 2:157-60.

Birnbaum, L.S. (1994). The mechanism of dioxin toxicity: Relationship to risk assessment. Environmental Health Perspectives. 102: 157-167.

Birnbaum, L.S. and DeVito, M., J. (1995). Use of toxic equivalency factors for risk assessment of dioxins and related compounds. Toxicology 105: 391-401.

Benjamini, E. and Leskowitz, S. (1991) Immunology. A short course. 2nd edition. New York: Wiley-Liss, Inc. p. 26

Burleson, G.R.; Lebrec, H.; Yang, Y.G.; Ibanes, J.D.; Pennington, K.N. (1996). Effect of TCDD on Influenza Virus Host Resistance in Mice. Fundamental and Applied Toxicology. 29: 40-47.

Calvert, G.M.; Sweeney, M.H.; Deddens, J.; Wall, D.K. (1999) Evaluation of diabetes mellitus, serum glucose, and thyroid function among United States workers exposed to 2,3,7,8- tetrachlorodibenzo-p-dioxin. Occupational and Environmental Medicine. 56(4):270-276.

Chao, W.Y.; Hsu, C.C.; Guo, Y.L. (1997). Middle-ear disease in children exposed prenatally to polychlorinated biphenyls and polychlorinated dibenzofurans. Archives of Environmental Health 52 (4): 257-262).

Crowther, J.R. ELISA: Theory and Practice. Series: methods in Molecular Biology. Vol 42. 1995. Totowa, NJ. Humana Press, Inc.

Cunningham, A.J. and Szenberg, S. (1968). Further improvements in the Plaque technique for detecting antibody-producing cells. Immunology. 14:599-601.

Davis, D. and Safe, S. (1988). Immunosuppressive activities of polychlorinated dibenzofuran congeners: quantitative SAR relationships and interactive effects. Toxicology and Applied Pharmacology. 94:141-149.

Davis, D. and Safe, S. (1990) Immunosuppressive activities of polychlorinated biphenyls in C57Bl/6 mice: structure activity relationships as Ah receptor agonists and partial agonists. Toxicology 63, 97-111.

De Heer, C.; Schuurman, H.J.; Vos, J.G.; and Van Loveren, H. (1994) Lymphodepletion of the thymus cortex in rats after single oral intubation of 2,3,7,8-TCDD. Chemosphere 29 (9-11):2295-2299.

De Krey, G.K. and Kerkvliet, N.I. (1995). Suppression of cytotoxic T lymphocyte activity by 2,3,7,8-TCDD occurs in vivo, but not in vitro, and is independent of corticosterone activation. Toxicology:97 (1-3): 105-112.

DeVito, M.J. (1994). Dose-response relationships in mice following subchronic exposure to 2,3,7,8-TCDD: CYP1A1, CYP1A2, estrogen receptor and protein tyrosine phosphorylation. Toxicology and Applied Pharmacology.124: 82-90.

DeVito, M.J.; Diliberto, J.J.; Ross, D.G.; Menache, M.G.; Birnbaum, L.S. (1997) Dose-response relationships for polyhalogenated dioxins and dibenzofurans following subchronic treatment in mice. I .CYP1A1 and CYP1A2 enzyme activity in liver, lung and skin. Toxicology and Applied Pharmacology 147:267-280.

DeVito, M.J.; Birnbaum, L.S.; Farland, W.H.; Gasiewicz, T.A.(1995) Comparisons of estimated human body burdens and TCDD body burdens in experimentally exposed animals. Environmental Health Perspectives 103(9):820-830.

Dooley, R.K. and Holsapple, M.P. (1988) Elucidation of cellular targets responsible for TCDD induced suppression of the antibody response: the role of the B lymphocyte. Immunopharmacology. 16:167-180.

Egeland, G.M.; Sweeney, M.H.; Fingerhut, M.A.; Wille, K.K.; Schnorr, T.M.; and Halperin, W.E. (1994) Total serum testosterone and gonadotropins in workers exposed to dioxin. American Journal of Epidemiology. 139 (3): 272-281.

Engvall, E. and Perlman, P. (1971) Enzyme-linked Immunosorbent Assay (ELISA) quantitative assay of Immunoglobulin G. Immunochemistry. 8:871-874.

EPA Health Effect Guidelines. OPPTS 870.7800. Immunotoxicity.

Feeley, M.M. and Grant, D.I. (1993) Approach to Risk Assessment of PCDDs and PCDFs in Canada. Regulatory Toxicology and Pharmacology18 (3):428-37.

Gehrs, B.C.; Riddle, M.M.; Williams, W.C.; Smialowicz, R.J. (1997) Alterations in the developing immune system of the F344 rat after exposure to 2,3,7,8 TCDD. I. Effects on the fetus and the neonate. Toxicology 122:219-228.

Gehrs, B.C.; Riddle, M.M.; Williams, W.C.; Smialowicz, R.J. ((1997) Alterations in the developing immune system of the F344 rat after exposure to 2,3,7,8 TCDD. II Effects on the pup and adult. Toxicology 122:229-240.

Gehrs, B.C., and Smialowicz, R.J. (1999) Persistent effects of delayed-type hypersensitivity in adult F344 rats after perinatal exposure to 2,3,7,8-TCDD. Toxicology 134(1): 79-88.

Gilman, A. and Newhook, R. (1991) An updated assessment of the exposure of Canadians to dioxins and furans. Chemosphere 23: 1661-1667.

Greenlee, W.F.; Dold, K.M.; Irons, R.D.; Osborne, T.(1975) Evidence for direct action of 2,3,7,8 TCDD on thymic epithelium. Toxicology and Applied Pharmacology 79:112-120.

Hanahan, D.J., and Ekholm, J.E. (1974) The preparation of red cell ghosts (membranes) Vol. XXXI in Fleischer, S., Packer, L. (Eds.) : "Methods in Enzymology: Biomembranes." New York: Academic Press. Pp. 168-172.

Hanberg, A.; Hakansson,H.; Ahlborg, U.G. (1989) "ED 50" values for TCDD-induced reduction of body weight gain, liver enlargement, and thymic atrophy in Hartley guinea pigs, SD rats, C57/bl6 mice and golden Syrian hamsters. Chemosphere 19:813-816.

Hanson, C.D.; Smialowicz, R.J. (1994) Evaluation of the effect of low-level 2,3,7,8-tetrachlorodibenzo-p-dioxin exposure on cell mediated immunity. Toxicology 88: 213-224.

Harper, N.; Conner, K.; Steinberg, M.; and Safe, S. (1994) An enzyme-linked Immunosorbent assay (ELISA) specific for antibodies to TNP-LPS detects alterations in serum immunoglobulins and isotype switching in C57BL/6 and DBA/2 mice exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin and related compounds. Toxicology 92: 155-167.

Harper, N.; Howie, L.; Connor, K.; Dickerson, R. and Safe, S. (1993) Immunosuppressive effects of highly chlorinated biphenyls and diphenyl ethers on T cell dependent and independent antigens in mice. Toxicology 85: 123-135.

Harper, N.; Steinberg, M. and Safe, S. (1996) Immunotoxicity of a reconstituted polynuclear aromatic hydrocarbon mixture in B6C3F1 mice. Toxicology 109:31-38.

Harper, N.; Connor, K.; Steinberg. M.; and Safe, S. (1995) Immunosuppressive activity of polychlorinated biphenyl mixtures and congeners: nonadditive (antagonistic) interactions. Fundamental and Applied Toxicology 27: 131-139.

Heyman, B.;(1984) An Enzyme-linked Immunosorbent assay for measuring Anti-sheep erythrocyte Antibodies. Journal of Immunological Methods.68: 193-204.

Holladay, S.D.; Lindstrom, P.; Blaylock, B.L.; Comment, C.E.; Germolec,D.C.; Heindell, J.J. and Luster, M.I. (1991) Perinatal thymocyte antigen expression and postnatal immune development altered by gestational exposure to TCDD. Teratology 44: 385-393.

Holsapple, M.P.; Snyder, N.K.; Wood,S.C.; and Morris, D.L. (1991). A review of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin induced changes in immunocompetence:1991 update. Toxicology 69(3): 219-55.

House, R.V.; Lauer, L.D.; Murray, M.J.; Thomas, P.T.; Ehrlich, J.P.; Burleson, G.R. and Dean, J.H. (1990) Examination of immune parameters and host-resistance mechanisms in B6C3F1 mice following adult exposure to 2,3,7,8-TCDD. Journal of Toxicology and Environmental Health 31:203-215.

Jerne, M.K.; Nordin, A.A.; and Henry C. (1963) The agar plaque technique for recognizing antibody-producing cells. In: Amos, B., Koprowski, H. (Eds.) Cell-bound Antibodies. Philadelphia: Wistar Institute Press.

Kerkvliet, N.I.; Steppan, L.B.; Smith, B.B; Youngberg, J.A.;Henderson, M.C.; Buhler, D.R. (1990) Role of the Ah locus in suppression of cytotoxic T lymphocyte activity by halogenated aromatic hydrocarbons (PCBs and TCDD): Structure activity relationships and effects in C57BL/6 mice congenic at the Ah locus. Fundamental and Applied Toxicology 14(3):532-541.

Kerkvliet, N.I. (1995) Immunological Effects of chlorinated dibenzo-*p*-dioxins. Environmental Health Perspectives. 103:47-53.

Kerkvliet, N.I. and Brauner, J.A. (1990) Functional analysis of APCs following antigen challenge: influence of 2,3,7,8- TCDD. 29th Annual meeting of the Society of Toxicology, Miami Beach, Florida. Feb. 12-16, 1990. The Toxicologist 10:289. (Abs. 1155)

Ladics, G.S.; Smith, C.; Elliot, G.S.; Slone, T.W.; Loveless, S.E. (1998). Further evaluation of the incorporation of an immunotoxicological function assay for assessing humoral immunity for hazard identification purposes in rats in a standard toxicology study. Toxicology 126: 137-152.

Lawrence, B.P.; Leid, M. and Kerkvliet, N.I. (1996) Distribution and Behavior of the Ah receptor in Murine Lymphocytes. Toxicology and Applied Pharmacology 138: 275-284.

Lindstrom, P.; Germolec, D.; Comment, C.; Luster, M.; Morrissey, R. (1990) Effects of gestational exposure to 2,3,7,8-TCDD on thymic lymphocyte populations. Teratology 41(5):575.

Lu, Y.C.; Wu, Y.C. (1985) Clinical findings and immunological abnormalities in Yu-Cheng patients. Environmental Health Perspectives 59:17-29.

Luebke, R.W.; Copeland, C.B. and Andrews. D.L. (1994) Assessment of host-resistance to Trichinella spiralis in mice following pre-infection exposure to 2,3,7,8-TCDD. Toxicology and Applied Pharmacology 125(1):7-16.

Luster, M.I. (1979) Laboratory studies on immune effects of halogenated compounds. Annals of the New York Academy of Science 320: 473-485.

Luster, M.I.; Boorman, G.A.; Dean, J.H.; Harris, M.W.; Luebke, R.W.; Padarathsingh, M.L.; Moore, J.A. (1980) Examination of the bone marrow, immunologic parameters, and host susceptibility following pre-and postnatal exposure to TCDD. International Journal of Immunopharmacology 2 (4): 301-10.

Luster, M.I.; Munson, A.E.; Thomas, P.T.; Holsapple, M.P.; Fanters, J.D.; White, K.L., Jr.; Lauer, J.D.; Germolec, D.R.; Rosenthal, G.J.; and Dean, J.H. (1988). Development of a testing battery to assess chemical-induced immunotoxicity. National Toxicology Program's guidelines for immunotoxicity evaluation in mice. Fundamental and Applied Toxicology Jan 10(1):2-19

Luster, M.I.; Germolec, D.R.; Clark, G.; Weigland, G.; Rosenthal, G.J. (1988) Selective effects of TCDD and corticosteroid on in vitro lymphocyte maturation. J. of Immunol. 140:928-935.

Luster, M.I.; Portier, C.; Pait, D.G.; Germolec, D.R. (1994) Use of animal studies in risk assessment for immunotoxicology. Toxicology 92:229-243.

Luster, M.I.; Portier, C.; Pait, D.G.; White, K.L.; Gennings, C.; Munson, A.E.; Rosenthal, G.J. (1992) Risk assessment in Immunotoxicology. I. Sensitivity and Predictability of Immune Tests. Fundamental and Applied Toxicology 18:200-210.

Luster, M.I.; Portier, C.; Pait, D.G.; Rosenthal, G.J.; Germolec, D.R.et al.(1993) Risk assessment in Immunotoxicology. II Relationship between Immune and Host Resistance Tests. Fundamental and Applied Toxicology 21:71-82.

Mazodier, P.; Motta-Ly, I.; Py, M.; Truffa-Bachi, P. (1977) Purification and characterization of a peripheral protein from the sheep erythrocyte. Journal of Immunological Methods. 14:41-50.

McConnell, EE. (1985) Comparative toxicity of PCBs and related compounds in various species of animals. Environmental Health Perspectives. 60: 29-33.

McGrath, L.F., Cooper K.R., Georgopolulos, P., and Gallo, M.A. (1995) Alternative models for low dose-response analysis of biochemical and immunological endpoints for 2,3,7,8 TCDD. Regulatory Toxicology and Pharmacology. 21:382-396.

Okey, A.B.; Riddick, D.S.; and Harper, P.A. (1994) Molecular biology of the aromatic hydrocarbon (dioxin) receptor. Trends in Pharmacological Sciences 15: 226-232.

Ott, M.G.; Zober, A.;Germann, C. Laboratory Results for selected target organs in 138

individuals occupationally exposed to TCDD. Chemosphere 29 (9-11): 2423-2437.

Poland, A. Glover, E., Kende, A.S. (1976) Stereospecific, high affinity binding of TCDD by hepatic cytosol. Evidence that the binding species is receptor for induction of aryl hydrocarbon hydroxylase. Journal of Biol Chem 251 (16):4936-46.

Poigner, H. and Schlatter, C. (1986) Pharmokinetics of TCDD in man. Chemosphere 15 (9-12):1489-1494.

Reggiani, G. (1978) Medical problems raised by the TCDD contamination in Serveso, Italy. Archives of Toxicology 40:161-168.

Rogan, W.J., Glad, B.C., Hung, K.L., et al. (1988) Congenital poisoning by polychlorinated biphenyls and their contaminants in Taiwan. Science 241:334-36.

Safe, S. (1990) PCBs, PCDDs, PCDFs, and related compounds: Environmental and Mechanistic Considerations which support the development of TEFs. Critical Rev. Toxicol 21:51-88.

Safe, S. (1992). Toxicology, structure-function relationship and human and environmental health impacts of polychlorinated biphenyls: Progress and problems. Environmental Health Perspectives 100: 259-268.

Safe, S. (1994) PCBs: Environmental impact, biochemical and toxic responses, and implications for risk assessment. Critical Review in Toxicology, 24(2):87-149.

Scheter, A.; Startin, J., Wright, C.; Kelley, M. et al.(1994) Congener-specific levels of dioxins and dibenzofurans in U.S. food and estimated daily dioxin toxic equivalent intake. Environmental Health Perspectives 102:962-971.

Scheter, A. and Olson, J.R. (1997)Cancer risk assessment using blood dioxin levels and daily dietary TEQ intake in general populations of industrial and non-industrial countries. Chemosphere 34: 1569-77.

Smialowicz, R.J.; DeVito, M.J.; Riddle, M.M.; Williams, W.C.; Birnbaum, L.S.(1994) Effects of TCDD on Humoral Immunity and Lymphocyte Subpopulations: Differences between Mice and Rats. Toxicology and Applied Pharmacology 124: 248-256.

Smialowicz, R.J.; DeVito, M.J.; Riddle, M.M.; Williams, W.C.; Birnbaum, L.S.(1997) Opposite Effects of PCB 153 and TCDD on the antibody response to Sheep Erythrocytes in Mice. Fundamental and Applied Toxicology 37:141-149.

Smialowicz, R.J., Williams, W.C., and Riddle, M.M. (1996) Comparison of the T-Cell Independent Antibody Response of Mice and Rats exposed to TCDD. Fundamental and

Applied Toxicology 32:293-297.

Strasser, A., Sonnek, U., and Niedermuller, H. (1997) Age-related changes in plasma IgM level after SRBC stimulation in the rat. Archives of Gerontology and Geriatrics 25:277-284.

Temple, L.; Kawabata, T.T.; Munson, A.E.; White, K.L. (1993) Comparison of ELISA and Plaque Forming Cell Assay (PFC) for measuring the response to SRBC in Rats and Mice treated with Benzo (a) pyrene or Cyclophosphamide. Fundamental and Applied Toxicology 21:412-419.

Temple, L. (1995) ELISA to Measure SRBC-specific IgM: Method and Data Evaluation. In: Burleson, G.R., Dean, J.R., and Munson, A.E. (Eds.): "Methods in Immuntoxicology. Vol. I." ©1995. New York: Wiley-Liss, Inc.

Thigpen, J.E., Faith, R.E., McConnell, E.E., Moore, J.A. Increased susceptibility to bacterial infection as a sequela of exposure to TCDD. Infect Immun.12 (6): 1319-1324.

Waldman, T.A.; Storober, W.; Blaese, R.M. (1979): in Merier E (ed): "Immunoglobulins." Washington, DC: National Academy of Science, pp 33-48.

Wood, S.C.; Jeong, H.G.; Morris, D.L.; Holsapple, M.P.(1993) Direct effects of TCDD on human tosilar lymphocytes. Toxicology 81:131-143.

Van Den Berg, M.; Birnbaum, L.S.; Bosveld, A.T.C.; Brunstrom, B.; Cook, P. et al. (1998) Toxic Equivalency Factors (TEFs) for PCBs, PCDDs, PCDFs for Humans and Wildlife. Environmental Health Perspectives. 106 (12):775-792.

Van Loveren, H.; Steerenberg, P.A.; Vos, J.G. (1995) Early detection of immunotoxicity: from animal studies to human biomonitoring. Toxicology Letters 77: 73-80.

Vos, J.G. and Van Loveren, H. (1995) Markers for immunotoxic effects in rodents and man. Toxicology Letters 82/83: 385-394.

Vos, J.G.; Moore, J.A.; Zinkl, J.G. (1973) Effect of TCDD on the immune system in laboratory animals. Environmental Health Perspectives 5:149-162.

Vos, J.G. Moore, J.A. (1974) Toxicity of TCDD in C57BL/6 mice. Toxicology Appl. Pharmacol. 29: 229-241.

Zober, A., Ott, M.G., Messerer, P. (1994) Morbidity follow-up study of BASF employees exposed to 2,3,7,8- TCDD after a 1953 chemical reactor incident. Occupational and Environmental Medicine. 51: 479-486.

APPENDICES

I. HEMOGLOBIN-FREE SHEEP RED BLOOD CELL (SRBC) MEMBRANE PREPARATIONS.

II. SRBC-SPECIFIC IgM ELISA PROTOCOL.

III. SRBC-PFC ASSAY.

I. HEMOGLOBIN-FREE SHEEP RED BLOOD CELL (SRBC) MEMBRANE PREPARATION

1. SCOPE OF APPLICATION

Hemoglobin Free SRBC membranes provide the antigen of interest for the SRBC-specific ELISA and are applied to microtiter plates (see protocol OP-NHEERL-H/ETD/ITB/RJS/IGMELISA/98-002-00.) Hemoglobin must be removed from the SRBC due to inherent reactivity of mouse serum with sheep hemoglobin. The following protocol describes how to prepare hemoglobin-free SRBC membranes for the SRBC -specific ELISA. This protocol was adapted from SOP #PFC/024, The Medical College of Virginia.

To isolate the SRBC membranes, defibrinated sheep blood is centrifuged using a swinging bucket rotor and washed with normal saline. After two washes, the buffy layer (WBC layer) is removed. The cells are then alternately washed with Tris-EDTA (which lyses the cells) and centrifuged using a fixed angle rotor until the hemoglobin is removed and the supernatant is clear. The membrane pellets are combined and filtered to remove the fibrous portion that may remain. The combined membranes are then placed in dialysis tubing and the suspension is dialyzed. Finally, the protein concentration of the dialyzed membrane solution is measured to provide an estimate of the isolated SRBC membranes.

2. PREREQUISITES

2.11 Chemical reagents:

- 10 mM Phosphate Buffered Saline (PBS), pH 7.4. Sigma Immunochemicals, catalogue # P3813, 2 packets.
- 200 ml Defibrinated Sheep Blood (Colorado Serum or Rockland)
- Lauryl Sulfate (sodium dodecyl sulfate), Sigma, catalogue # L4509
- Ethylenediaminetetraacetic acid (EDTA), Sigma, catalogue # E1644
- Trizma ® hydrochloride, Sigma, catalogue # T6666
- Deionized water (from lab)
- Spectra/Por ® Membrane MWCO:12-14,000. Catalogue # 132678
- BCA Protein Assay Reagent Kit. Catalogue # 23225

2.1.2 Equipment and supplies:

- Immulon-2 ELISA microtiter plate (flat-bottom), Dynatech Labs, Chantilly, VA
- Corning 200 pH meter
- IEC PR-7000 centrifuge with swinging bucket
- Sorvall Ultracentrifuge
- Glass Bottles with screw caps: 1 L (3), 500 ml (1)
- Stir bars
- Magnetic stir plate
- Glass beakers: 1L (1), 500 ml (1)

- 1 Liter Graduated cylinder (plastic or glass)
- Bio-Freeze Vials, 5 ml, Costar, catalogue # 2051
- 8-10, 40 ml round bottomed centrifuge tubes able to fit in both centrifuges
- Timers

2.2 Training Requirements
New Personnel will receive on the job training from lab personnel familiar with this procedure.

3. Cautionary Notes or Special Considerations

Although sterility of the solutions described in this procedure is not required, it is important to examine all of the solutions for contamination and to keep track of the dates of preparation.

The EPA Laboratory Safety Training course is required.

Almost all of the chemicals used in the procedure are classified as irritants, and the remainder as harmful or toxic. Therefore, it is required that laboratory personnel wear the appropriate personal protective equipment (i.e. lab coat, gloves, safety glasses) when preparing solutions. In addition, it is advisable to wear a particle mask when measuring SDS as the dust should not be inhaled.

4. PROCEDURE

4.1 Preparation of Solutions

Warning: PBS and Tris-HCl are classified as irritants to the eyes, respiratory system and skin. EDTA and SDS are classified as harmful by ingestion and harmful by inhalation, respectively. In case of contact with eyes or skin for any of these chemicals rinse immediately with water and seek medical advice. In case of inhalation exposure of SDS or ingestion of EDTA, seek medical advice immediately. Clean up spills and discard waste as described in the EPA/RTP Safety manual regarding the disposal of hazardous waste.

4.1.a The following solutions can be made up prior to, or the day of the experiment: (sterility is not necessary)

4.1.a.1 **Normal saline (0.85%)**

1. Add 2 sodium chloride tablets to 400 ml deionized water.
2. Mix at room temperature until the tablet is dissolved. Store at 2-8 °C in 500 ml glass bottle.

4.1.a.2 **5 mM Tris-HCL with 1 mM EDTA**

1. Weigh out 610 mg Tris-HCl and 370 mg EDTA.
2. Add to 800 ml deionized water and stir until dissolved.
3. Adjust pH to 7.6, and QS to 1 L with deionized water. Store at 2-8 °C.

4.1.a.3 **0.1 % SDS in PBS**

1. Add 1 packet of PBS to 1L deionized water. Stir until thoroughly mixed.
2. Add 1 gram of SDS to 1 L of PBS, stir until thoroughly mixed.
3. Store 0.1 % SDS at room temperature for use during dialysis. **Do not refrigerate or the SDS will precipitate out of solution.**

Note: at least 2L is needed for changing the solution twice over 24 hours.

4.2 **Hemoglobin-Free Sheep Red Blood Cell (SRBC) Membrane Preparation**

1.Approximately 200 mls of defibrinated sheep blood containing no Alsever's should be removed via sterile technique from the bottle. We have successfully used sheep blood from Rockland and Colorado Serum in this technique.

2. Aliquot approximately 25 mls of sheep blood into eight 40 ml, round bottomed centrifuge tubes. You may adjust the total volume of sheep blood to the number of centrifuge tubes available. These centrifuge tubes must be compatible for later use in a high or ultra speed centrifuge.

3. Centrifuge using swinging bucket rotor at 725 x g (1900 rpm setting for the centrifuge in room K 214) for 15 minutes at 4 °C. Aspirate the supernatant carefully as the pellet is difficult to distinguish from the supernatant.

4. Fill each tube with normal saline and mix thoroughly by pipetting up and down. Be sure to resuspend the entire pellet. Centrifuge under the same conditions as detailed in Step 1, and then aspirate the supernatant and fill each tube with normal saline.

5. Resuspend the pellet and centrifuge again. After this wash, look for the "buffy" layer (the layer of white cells). This layer will be difficult to see at first, but can be visualized by looking down the tube from the top, or from the side. The layer appears wrinkled.

6. Remove the buffy layer with a pipet or with vacuum suction. Don't be surprised to lose red blood cells: it is better to remove more RBCs than to leave the buffy layer intact.

7. Wash the cells with normal saline a third time, centrifuge and aspirate the

supernatant.

8. Add enough Tris-EDTA to the pellet in each tube to fill the tube. This solution will lyse the cells, facilitating removal of hemoglobin and cellular contents.

9. Using a fixed angle rotor, centrifuge the lysed cells at 25,000 x g for 30 minutes at 4°C. (The setting for the ultracentrifuge in room K-215 is 14800 rpm. If the pellets seem very diffuse, increase the setting to 15800 rpm.) Remove the supernatant carefully because the pellets will be difficult to see due to the dark color of the supernatant.

10. Repeat the washes with Tris-EDTA as many times as needed until the supernatant is clear. After each wash, try to resuspend the entire pellet in freshly added Tris-EDTA. The dark fibrinous portion may become stuck to the sides; however the white portion of the pellet contains the cell membranes.

11. The pellets may be refrigerated overnight at 4°C if all of the washes cannot be completed in one day. In fact, overnight storage seems to increase the SRBC yield (as estimated by protein content).

12. Once most of the hemoglobin has been removed, all of the pellets are combined. Each pellet is solubilized in approximately three times the pellet volume of 0.1% SDS/PBS . Pool the solutions and filter through 1-2 layers of ordinary gauze to remove the fibrinous material.

13.Soak a section of Spectra/ Por 2 dialysis tubing in distilled water for about 10-15 minutes. Check the tubing for tears or leaks by first filling with distilled water, and be sure that you have enough tubing for dialysis.

14. Pipet the pooled solution into the tubing after clamping one end with the orange clamp, and then clamp the top of the tubing. It is best to divide the total volume of the pooled solution in half and cut the tubing into equal sizes. This allows the dialysis tubing to fit into a 1-Liter beaker and immersed in 0.1% SDS/PBS.

15. Dialyze the suspension for @ 24 hours against 0.1%SDS/PBS at room temperature with stirring. Change the 0.1% SDS/PBS twice during this time period.

4.3 BCA Protein Assay

4.3.a The BCA Protein Assay Instruction Manual is attached. Use the protocol on page 7, "Microtiter Plate Protocol" to measure the concentration of the dialyzed solution at the correct wavelength (at or near 562 nm). The total volume is usually around 20-30 mls, with a protein concentration of 0.7-1.5 mg/ml. Aliquot the solution into the Bio-Freeze Vials (Costar) at a concentration of 0.5-1.0 mg/ml depending on usage and store at -20 °C. The SRBC membrane prep is stable at -20 °C for up to a year.

4.4 Quality Control

Clean glassware, pipets and other laboratory equipment is to be used for all solutions. Chemicals are dated upon receipt and stored appropriately. Solutions are monitored for contamination and are dated.
Sheep blood is used within 3 weeks of delivery. Maintenance and upkeep of centrifuges occurs in a timely manner to ensure optimal performance. The BCA protein assay incorporates negative (PBS) and positive (Bovine Sera Albumin) controls for quality control.

4.6 Evaluation Control

Consistent membrane prep protein concentrations within the range of 0.5-1.5 mg/ml demonstrate that SRBC membranes have been isolated. Further testing uses the SRBC IgM ELISA and a defined internal standard to detect successful antigen-antibody binding.

4.7 Corrective Action

If the protein concentration does not fall within the specified parameters, repeat the BCA Assay using fresh substrate. If the protein yield continues to be lower than expected, and/or no antibody binding is detected via ELISA, repeat the membrane preparation procedure with fresh SRBC.

4.8 Record keeping

A log is kept for the dates of receipt of the sheep blood. Protein concentrations and dates of preparation are written on the freezer vials for each SRBC membrane prep. This information is also recorded in a notebook.

4.9 Calculations

N/A

5 References

Temple et al., (1995): ***ELISA to Measure SRBC Specific Serum IgM: Method and Data Evaluation.*** *Methods in Immunotoxicity.* Vol 1.Burleson, G., Dean, J., and Munson, A.E., (eds.) © 1995 Wiley-Liss, Inc. New York, NY. pp 137-157.

Temple et al., (1993): ***Comparison of ELISA and plaque-forming cell assays for measuring the humoral immune response to SRBC for rats and mice treated with benzo(a)pyrene or cyclophosphamide***. *Fundam Appl Toxicol* 21:412-419.

II. SRBC-SPECIFIC IgM ELISA PROTOCOL

1. SCOPE OF APPLICATION

The Sheep Red Blood Cell (SRBC) -Specific IgM Enzyme Linked Immunosorbent Assay (ELISA) measures the levels of serum IgM specific to SRBCs. The ELISA technique introduced by Engvall and Perlman (1971) involves binding of antigen to a solid matrix and subsequent binding of a detectable marker. For the SRBC specific ELISA, SRBC membrane preparations provide the antigen of interest. (The protocol to prepare the membrane solution is described elsewhere.) Animals are immunized with the optimal concentration of SRBCs and at the peak response day, the sera are collected. The antigen (SRBC membrane prep) is applied to microtiter plates, and the binding of serum IgM anti SRBC is measured via detection with the appropriate secondary antibody and addition of substrate resulting in a characteristic color change. Measurement of the absorbance of this final solution via spectrophotometer at 410 nm allows for quantitation of serum levels of SRBC specific IgM.

It should be noted that the immunization conditions (i.e., concentration of SRBC to inject, kinetics study to determine optimal titer, and production of "internal standard" sera) should be optimized to detect possible strain differences. The protocol described below was utilized in this laboratory with male Sprague -Dawley rats and female B6C3F1 mice as the test animals. These animals were immunized as detailed in EPA protocol OP/NHEERL-H/ETD/ITB/RJS/PFCSRBC/96-00-90. Kinetics studies for these animals revealed optimal production of anti-SRBC IgM on Day 5 or 6 post-immunization in the rat, and on Day 5 post-immunization in the mouse in agreement with data presented by Temple et al (1993). The procedure reported below has been modified from the Dupont-Haskell Laboratory protocol, from SOP#PFC/024, Medical College of Virginia, and from Temple et al (1993) .

2 PREREQUISITES

2.1.1 CHEMICAL REAGENTS

- Immulon-2 ELISA microtiter plates, (flat-bottom), Dynatech Labs, Chantilly,VA
- 10 mM Phosphate Buffered Saline (PBS), pH 7.4. Sigma Immunochemicals, catalogue
  # P3813 1 packet.
- Hemoglobin-free sheep red blood cell (SRBC) membranes preparation as described in attached protocol; diluted to 1 mg/ml (protein) in PBS.
- Affinity Purified goat anti-rat IgM-HRP (μ) (rat secondary antibody), Accurate Chemical and Scientific Corp., Westbury, NY; or Affinity Purified goat anti-mouse IgM -HRP (μ) (mouse secondary antibody), Accurate Chemical and Scientific Corp., Westbury, NY.
- Nonfat Powdered Milk (local grocer)
- Polyoxyethylenesorbitan Monolaurate (Tween 20), Sigma, catalogue #P-7949, Lot #87HO992

- Distilled water, Gibco BRL, catalogue #15230-147, Lot #1014933
- 2,2' Azino-bis (3-Ethylbenzthiazoline-6-Sulfonic Acid) Diammonium Salt, Sigma, catalogue # A-994, Lot #76H8911
- Phosphate-citrate buffer with Urea Hydrogen Peroxide Tablets, Sigma, catalogue#P-9305, Lot # 27H8913
- Internal Standard (Serum from control mouse or rat)[1]
- Test Serum (mouse or rat)[1]

2.1.2 EQUIPMENT AND SUPPLIES

- Costar Multichannel Pipettor, 12 tip
- Costar Disposable pipet tips (200 ul) Cat # 4953
- Stir bars
- Plastic Container, 10 L (able to be attached to Plate washer)
- Glass beakers: 200ml(2), 1000ml(1), 250 ml(1)
- Costar Reagent Reservoir, Cat# 4870
- Corning 50 ml Centrifuge Tubes, Cat #25325-50
- Test tube racks
- Sarstedt 75 X 12mm test tubes,Cat #55.476
- Magnetic stir plate
- Nunc-Immuno Wash 12 (12 well plate washer) or equivalent and plastic tubing
- SpectraMax 250 plate reader or equivalent
- Softmax software or equivalent
- Timers

2.2 Training Requirements

New personnel will receive on the job training from lab personnel familiar with this procedure.

3 CAUTIONARY NOTES OR SPECIAL CONSIDERATIONS

Accurate pipetting skills are essential to successful assays.

Although sterility of the solutions described above is not required, it is important to examine all solutions for contamination, and to keep track of the dates of preparation.

The EPA Laboratory Safety Training course is required.

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[1] **Note**: Do not heat inactivate the sera. This has been shown to result in non-specific binding.

A number of the chemicals used in the procedure are irritants. Therefore, it is recommended thatlaboratory personnel were the appropriate personal protective equipment (i.e., lab coat, gloves, safety glasses) when preparing the solutions

4 PROCEDURE

4.1 Preparation of ELISA Solutions

WARNING

PBS, Phosphate-citrate buffer with urea hydroxide, and Peroxidase substrate are classified as irritants to the eyes, respiratory system, and skin. In case of contact with eyes or skin, rinse immediately with water and seek medical advice. Clean up spills and discard waste as described in the EPA/RTP Safety manual regarding the disposal of hazardous waste.

4.1.a **The following solutions should be prepared prior to the day of the assay:**

4.1.a.1 **Phosphate Buffered Saline**
[*Sterility is not necessary.*]

1. Place the contents of 1 PBS package into a glass beaker. Add 1000 ml of deionized water. Gently mix the solution by stirring until thoroughly dissolved. Adjust pH to 7.4.

2. Store in the refrigerator (2-8 C) for up to 3 months. Can be used at room temperature or straight from the refrigerator.

4.1.a.2 **Wash Solution: 0.05% Tween 20 in Deionized Water**
[*Procedure to prepare 10 liters; sterility is not necessary.*]

1. Place 10 liters of deionized, glass distilled water in plastic jug. Note: This does not have to be the Gibco brand water and can be lab grade water.

2. Remove 5 ml of the water and add 5 ml of Tween 20.

3. Store at room temperature for up to two weeks. The solution can be used at room temperature or straight from the refrigerator.

4.1.a.3 Dilution Buffer: 0.05 % Tween 20 in PBS
[*Procedure to make 200 ml; sterility is not necessary.*]

1. Place 200 ml of PBS in a 250 ml glass beaker.

2. Remove 100 μl of the PBS and add 100 μl of Tween 20. Mix by gently shaking.

3. The Dilution Buffer can be stored and used at room temperature for up to two weeks.

4.1.b <u>Prepare the following solution the day before the assay:</u>

4.1.b.1 Blocking Solution: 4.0% Nonfat Milk
[*Procedure to prepare 100 ml; sterility is not necessary.*]

1. Weigh out 4.0 g of non-fat powdered milk and place in a 200 ml glass beaker.

2. Add 100 ml of distilled water (Gibco BRL). Add a stir bar and gently mix until the powdered milk dissolves.

3. Store the Blocking Buffer at 2-8 C until the day of the assay. Prior to application, gently mix the solution again; solution can be used at room temperature or cooler. Dispose of remaining solution after assay.

4.1.c <u>Prepare the following solution the day of the assay, 1 hour prior to use:</u>

4.1.c.1 Phosphate -Citrate Buffer with Urea Hydroxide Peroxide Solution
[Procedure to prepare 100 ml (sterility is not necessary)]:

1. Place 1 tablet of phosphate-citrate buffer with urea hydrogen peroxide into a 200 ml glass beaker.

2. Add 100 ml of distilled water (Gibco BRL). Add a stir bar and mix gently until the tablet is dissolved.

3. Let the solution stand at room temperature until usage. After assay, discard the remaining solution.

4.1.d <u>Prepare the following solution the day of the assay, immediately prior to application</u>

4.1.d.1 Peroxidase Substrate

[2,2 Azino-bis (3-Ethylbenzthiazoline-6-Sulfonic Acid) Diammonium Salt; Procedure to prepare 50 ml (sterility is not necessary)]:

1. Place 50 ml of Phosphate Citrate buffer with urea hydrogen peroxide solution into a 50 ml screw-top conical.

2. Add one 10 mg tablet of the 2,2'-Azino-bis (3-Ethylbenzthiazoline-6-Sulfonic Acid) Diammonium Salt to the solution. Mix by inversion at room temperature until the tablet is dissolved. It is recommended to use 50 ml of this solution at a time to prevent overdevelopment of substrate prior to application.

Optimally, this solution is used immediately after preparation; however if more than one plate is being prepared, substrate can be used up to 15 minutes after preparation. Extra solution is to be discarded after use.

4.2 Performing the Optimization Study: Microtiter Plate Conditions

It is recommended that prior to testing the serum of interest by ELISA an optimization study for plate conditions be conducted. The purpose of this procedure is to optimize the following conditions for the SRBC IgM Specific ELISA: SRBC membrane concentration for coating the bottom of the plates, secondary antibody concentration, and starting serum dilution for the test serum. Thus, this "preliminary" assay will account for the variability which exists between different batches of the above components as well as improving the reliability of the investigator's technique.

4.2.a Coating of Microtiter Plate with SRBC Membrane Preparation

1. Plates must be coated with SRBC membrane prep and refrigerated for a minimum of 16 hrs **prior to the day of the assay** and may be used for up to 1 week after coating/refrigerating.

2. Obtain an aliquot of the SRBC membrane prep solution from the freezer. (The stock solution of SRBC membrane prep should have been prepared previously as described in OP-NHEERL-H/ETD/ITB/RJS/_____/_____ and stored in aliquots of 1 mg/ml (protein) at -20°C.

3. Thaw the SRBC membrane prep and dilute to the following concentrations in PBS: 16.0 μg/ml, 8.0 μg/ml, 4.0 μg/ml, 2.0 μg/ml and 1.0 μg/ml. Be sure to make enough of each solution to allow for excess. These concentrations will correspond to 2.0 μg/well, 1.0 μg/well, 0.50 μg well, 0.25μg/well, and 0.125 μg/well when 125 μl is pipetted per well, as shown in Figure 1. It is recommended that duplicates for each

concentration of the SRBC membrane prep be used to insure greater accuracy.

4. Vortex the solutions prior to application to the plates.

5. Using the reagent reservoirs and the 12 well Multichannel Pipettor, add 125 μl per well of each dilution into the designated wells.

CAUTION

When pipetting all solutions, be sure to check for air bubbles in the pipette tips, and check to insure that the volumes aliquotted are the same for each well. Be careful not to leave air bubbles in the wells.

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6. Wrap the plates in Saran Wrap to minimize evaporation and incubate in the refrigerator overnight.

4.2.b ELISA for Determining Optimal Conditions/Endpoints

4.2.b1 Wash and Block Step

1. Hand decant the plates by quickly snapping the plate over with a flick of the wrist and dry by gently blotting the inverted plate on a paper towel.

2. Using the plate washer, wash the plate 3 times with the Wash Solution, then blot again on a paper towel.

3. Using the multipipettor and a disposable plastic trough, coat the bottom of each well with 200 μl of Blocking Buffer.

4. Cover the plate and incubate at room temperature for 1 hour.

5. While the plates are incubating, take the Internal Standard out of the freezer, thaw and vortex the samples. For the optimization study, it is recommended to start with a 1:8 dilution of the Internal Standard in Dilution Buffer.

6. After the incubation time is complete, decant the Blocking Buffer, and wash as described above.

4.2.b.2 Addition of Control Serum

1. Add 100μl of PBS to each well, excluding those wells which will be used as

the first wells in Column 2. These wells in Column 2 which will be the first wells of the serial dilution. Column 1 wells are used as controls and receive no serum, only PBS.

Note: It is easiest to use the mutlichannel 12 well pipettor with 12 tips, remove the second tip and load with 100 μl PBS. This will leave Column 2 empty for addition of test sera.

2. Add 200 μl per well of the diluted serum sample to Column 2 wells, then initiate the serial dilution by taking 100 μl from Column 2 wells, add it to the Column 3 wells, then gently pipet up and down a constant number of times to mix well. Proceed in this manner (i.e. take 100 μl from Column 3 wells and add to Column 4 wells) until you have reached the end of the row. The last dilution should be 1: 8192 as shown in Figure 1.

3. Discard the liquid held in the pipettor tips at the end of the serial dilutions. A final volume of 100μl should be contained in all wells.

4. Incubate the plates for 1 hour at room temperature.

5. Near the end of the incubation period, thaw the secondary antibody and dilute to the desired concentration in Blocking Buffer.

6. Hand decant the plate, then wash 3 times with Wash Solution.

Note:

1. Storage of the secondary antibody.
The secondary antibody can be reconstituted in distilled water to the specifications of the manufacturer and stored at 2-8 C for up to 6 months. For extended use (longer than 6 months) it is recommended that the reconstituted secondary antibody be stored in 50% glycerol in the freezer (i.e. 1:1 antibody/100% glycerol.)

2. Titration of the Secondary Antibody.
The secondary antibody will also have to be titrated as the activity will vary from batch to batch from the manufacturer. A new plate will be made, holding all other factors constant, for each concentration of secondary antibody to determine optimal antibody concentration. It is recommended to begin by testing the following dilutions for HRP conjugated goat anti-mouse and anti-rat IgM: 1:5000, 1:3000, 1:2000, 1:1500, 1:1000. See Figure 2 for an example of plate set up.

4.2.b.3 Addition of Secondary Antibody

1. Add 100μl of the secondary antibody solution to each well of the microtiter plate.

2. Cover plate and incubate for 1 hour at room temperature.

3. Hand decant the plate and wash three times with Wash Solution.

4.2.b.4 Addition of Peroxidase Substrate.

1. Add 100 μl of peroxidase substrate to each well, then cover the plate.

2. Incubate at room temperature for 45 minutes.

3. Insert plate into the SpectraMax 250 plate reader (or equivalent) and read at 410 nm wavelength.

4.3 Data Analysis of the Results from the Optimization Study

We use Softmax software to analyze the results of the assay. A copy of the Softmax Software manual is located in room K216. Be sure to read the sections on Computer Systems Requirements and transferring Macintosh/ PC files.

4.3.a Creating a Template

After reading the plate and saving the data as appropriate, a template plate must be designed to allow the program to accurately analyze the data. Click on the Template Button shown in Figure 3. The Template window shown in Figure 4 will open. Click on the Group pop-up frame (Figure 5) and choose "New" and then go to Column Format and choose "Unknowns/Dilution". Still within this window, go to "Name" and type in the appropriate name (i.e. group #1= 0.125μg/well SRBC) This designates a group of wells that have the same properties (i.e. SRBC membrane coating) but differing dilutions of sera. Next assign the blanks by clicking on the well that has only PBS, go to the "Sample" pop-up window and choose "BL". The program will automatically subtract the blanks from the test wells. Moving to the next well, click on the well and type the dilution into the "Dilution Factor" window (i.e. for a 1:8 dilution, type in "8"). Continue in this manner until all wells have been assigned the group and dilution. Click "O.K" to accept the final template. See Figure 6 for an example of a Template for an Optimization Study.

Note: Once you have set up a template, you can copy the template from file to file, avoiding having to set up a new template each time.

4.3.b Graphing

Return to the original window and click on Edit and choose the New Graph option. In the pop-up window (Figure 7) under "Plot Name", type in a group name. In the Group pop-up window choose the same group. Select the X and Y values (X=dilution, Y=Values, or MeanValues if you have averaged duplicates). Go to the Fit button and choose "4 Parameter Curve". This should give the best fit when you include all data points. The sigmoid curve should have a plateau region at the lowest (most concentrated) dilutions, and then have a somewhat linear region (slope around -1.0) before tapering off at the highest dilutions To

choose the optimal conditions, look for the SRBC membrane concentration which gives a good sigmoid curve with low background values (Blanks) and the highest OD readings for the control serum. At the highest concentration of SRBC you may find that the ODs are lower than the other test concentrations. This reflects saturation of the binding capabilities of the antigen with antibody. For the plates that you have tested secondary antibody concentrations, plot the results and choose the dilution which gives good ODs with low background activity. If there is no discernible or very slight differences among the test concentrations, choose the lowest concentration of SRBC and secondary antibody in order to conserve reagents. (See Figure 8 as an example). The sera dilution that is at the end of the plateau region and in the beginning of the linear region is an appropriate starting point for the dilution of the internal standard for the ELISA.

4.4 ELISA for Experimental (Test) Sera Analysis and Data Analysis of the Results

1. After the optimal plate conditions have been determined, the ELISA assay with the test serum should progress through the same steps as outlined above in Sections 4.2.1. through Section 4.2.2.d., except that the entire plate will have been coated with the optimal SRBC membrane prep concentration and the secondary antibody will be used at the optimal dilution.

2. The data analysis method for analyzing the results of the ELISA for the test sera is slightly different than the method that is used to determine the various optimal endpoints. Results are reported as the titer of each test sera to give an OD of 0.5. In order to calculate this titer, set up the template and identify each "group" of serum sample by the number of the test animal or as the internal standard. All "blanks" should be identified and will automatically be subtracted out by the software. Plot the data as described above, but choose log-log under the Fit window, and identify the linear portion of the curve. Select at least one point above 0.5 OD and two or three values below 0.5 OD in order to obtain a slope approaching -1.000. (The slope should lie between -0.800 and -1.200). To calculate the slope of the best-fit line, go to the plate window and "mask" those wells that lie on either side of the linear portion. (See Figure 9). This will tell the program to eliminate those values from the calculation of the slope. Once the best -fit line is established, interpolate at 0.5 OD. This can be done by creating a new column for data for each test serum, and then inputting the following equation: interpx(plot#__@ graph#_, 0.5). Fill in the plot number and graph number in this equation for each test serum. This represents the "titer" of the sample.

4.5 Quality Control

Use of the internal standard for all ELISAs is required to detect interplate variation and inter-experiment reproducibility. Particular attention is paid to the "Blanks" (wells that contain PBS only) to detect non-specific binding. Blanks should routinely register an OD of 0.150 or less, ideally less than 0.100.

Out-dated reagents are to be disposed appropriately and in a timely manner. Except where noted, only clean disposable solution containers, pipets, and tips are to be used.

4.6 Evaluation Criteria

Consistent titers for the internal standard across experiments serves as the evaluation criteria for the successful application of the SRBC-Specific IgM ELISA for analysis of sera from experiments.

4.7 Corrective Action

Repeat ELISA on experimental serum samples if internal standard varies more than 0.50 of the Log_2 value of the dilution to give 0.50 OD.

Example: The Internal Standard titer to give 0.50 OD is 200 (1:200 dilution of pooled serum).
$\text{Log}_2(200) = \text{Log}_{10}(200) / 0.301 = 7.64$. Therefore, values on subsequent plates for the Internal Standard are accepted between 7.14 and 8.14.

Repeat ELISA on experimental serum samples if the blanks are above 0.150. High background values are indicative of non-specific binding.

4.8 Record keeping

Softmax software files are created when each plate is read on the plate reader and analyzed, and stored on the hard drive and as a backup copy on a floppy disk. A hard copy should be generated and kept in a notebook with other information. Information required in the notebook includes the date of the assay, optimal conditions for the ELISA, the membrane preparation used, and the sera of interest.

4.6 Calculations

SEE Sections 4.4 and 4.7

5 QUALITY CONTROL RATIONALE

This protocol includes several quality control steps. An Internal Standard (normal rat or mouse sera) is used for every ELISA to detect interplate variation and interexperiment reproducibility. Internal standards are used as a reference point allowing the investigator to assure that all components of the ELISA are at optimal conditions.

Blank wells containing only PBS are used for every ELISA experiment. Blanks are included to assess background reactivity to secondary antibody and to detect possible contamination of solutions.

Maintenance of working solutions and record-keeping of dates is necessary to prevent contamination and loss of reactivity of secondary antibodies.

6 References

Temple et al. ELISA to Measure SRBC Specific Serum IgM: Method and Data

Evaluation. In ***Methods in Immunotoxicity. Vol I.*** (G. Burleson, J. Dean and A. Munson, eds.© 1995) Vol I, pp.137-157

Crowther, J.R. (1995) ***ELISA, Theory and Practive.*** Humana Press Inc. Totowa, NJ, pp.1-50, 63-79.

Engvall, E., and Perlmann, P. (1971) Enzyme-linked immunosorbent assay (ELISA) quantitative assay of Immunoglobulin G. ***Immunochemistry*** 8, 871-874.

7 APPENDICES

EXPERIMENT Optimization of SRBC membrane prep DATE August 13 98

Pretreatment SRBC membrane 1.00, 0.500, 0.250, 0.125 ug/well Antibodies Goat Anti Rat Other

Antigen SRBC membrane

Blocking 4% Non Fat milk

Rat Serum (pooled)
Is Internal Standard

Goat Anti Rat
Ism HRP
1:2000 in Blocking Buffer

1:2000 2° Ab.

SRBC (ug)/well		1	2	3	4	5	6	7	8	9	10	11	12
0.125	A	BL	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096	1:8192
	B												
0.250 PLATE	C												
	D												
0.500	E												
	F												
1.00	G												
	H												

(PBS) (serial dilution of Internal Standard)

		1	2	3	4	5	6	7	8	9	10	11	12
	A												
	B												
	C												
PLATE	D												
	E												
	F												
	G												
	H												

EXPERIMENT Optimization of 2° Ab Dilution DATE August 15, 78

Pretreatment SRBC membrane Prep 0.25 ug/well Antibodies Goat Anti Rat Other

Antigen SRBC membrane IgM HRP

Blocking 4% Non Fat milk 1:1500 1:2000,

Rat Serum (pooled) 1:3000, 1:5000

in internal Standard in Blocking Buffer

0.250 ug/well SRBC membrane

Ab

	1	2	3	4	5	6	7	8	9	10	11	12
A	BL	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096	1:8192
B												
C												
D												
E												
F												
G												
H												

1500

2000 PLATE

3000

(RBC) (Serial dilution of internal Standard)

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Figure 3

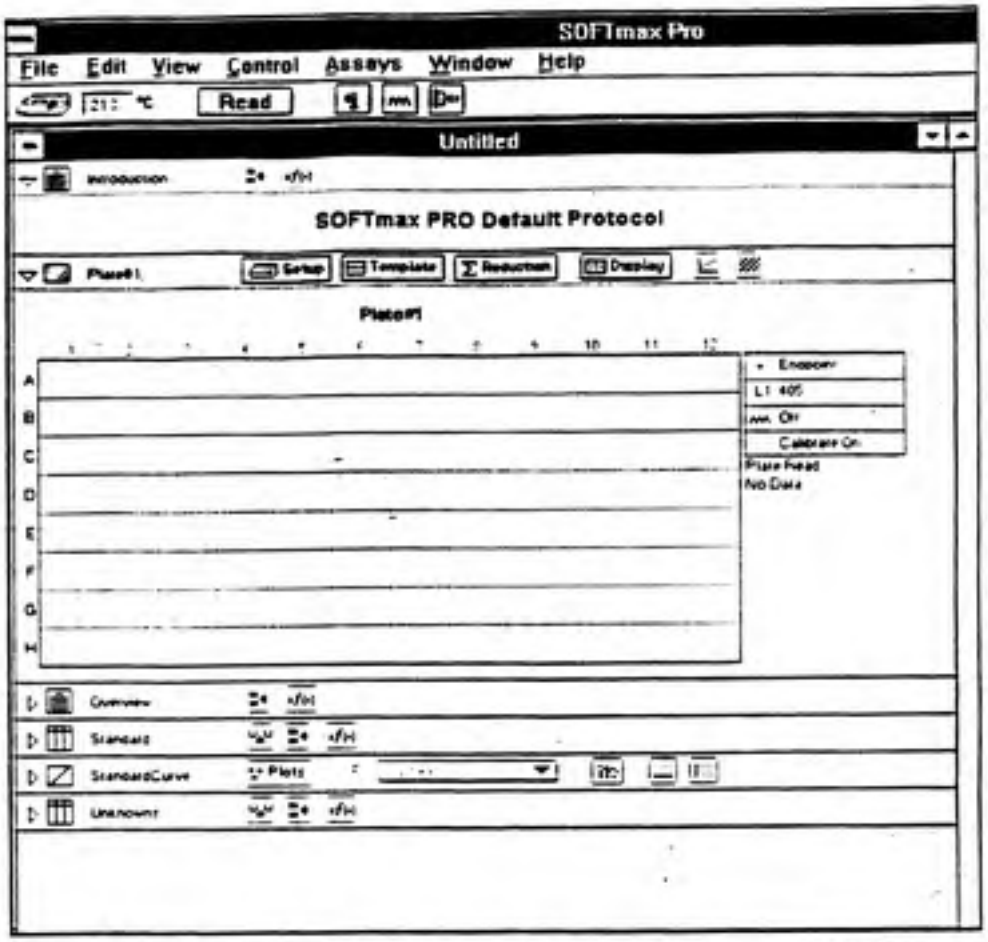


Figure 2.10: SOFTmax PRO (Windows Version) Default Protocol Untitled Window

Communicate with the MAXline Instrument

Before continuing, check that the instrument icon in the upper left-hand corner of the window appears without an "X." If the icon has an "X" through it, check whether the MAXline instrument is turned on. If it is, then check the SOFTmax PRO Preferences settings (see Setting Preferences below) to ensure that the proper communications port (modem or printer for the Macintosh, COM1 or COM2 for the PC) is selected. If the port setting is correct, the MAXline instrument is on, and communication is still not functioning properly, quit SOFTmax PRO, turn off power to both the computer and the MAXline instrument, and check that the cable connections between the instrument and computer are secure. Turn both machines on again and restart SOFTmax PRO. If an "X" still appears over the icon, refer to Chapter 6, "Errors and Troubleshooting," for more information.

Figure 3.28: Group Settings Dialog Box for the Group Named "Patient#1"

If no group is active or selected when the Edit button is clicked, default group settings will be used: the group name will be created as "Group#X" (where "X" is a sequential number that begins with "1" and increments to the next number with each subsequent new group) using the default group column format, "Basic." The figure below shows the Group Settings dialog box for such default settings.

Figure 3.29: Group Settings Dialog Box Using Default Settings

You can enter a new name and any appropriate sample descriptors for a new group. The checkbox next to "Include in Report" will be selected by default; you can disable this feature if you do not want information about the new group to be included in the printout after a reading.

The choices in the area labeled "Column Format" define the type of group from which the defaults for the new group are taken: Basic, Standard, Unknown, and

Figure 6

Example of Template set up in Soft max

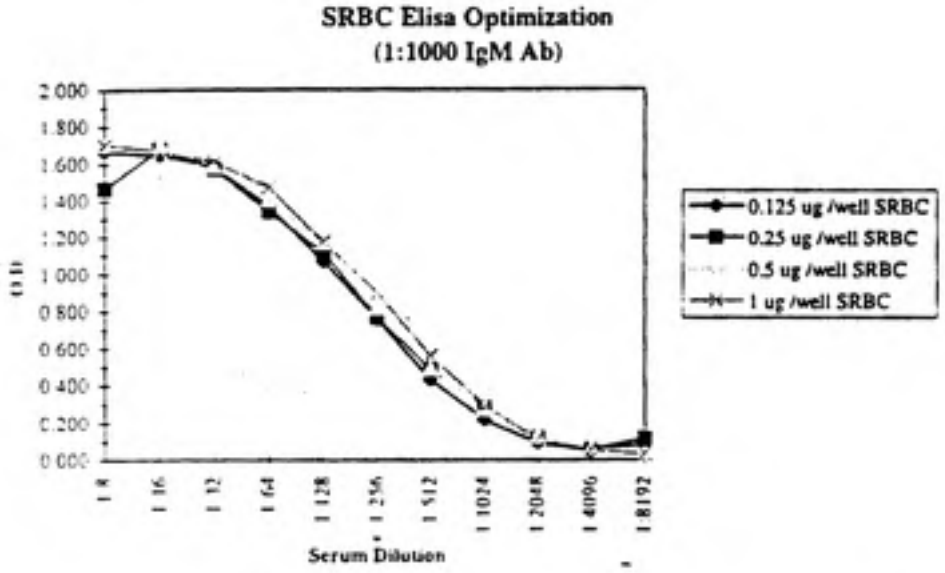
	1	2	3	4	5	6	7	8	9	10	11	12
A	BL	.1201	.1202	.1203	.125 ug/well .1204	.1205	.1206	.1207	.1208	.1209	.1210	
B	BL	.2501	.2502	.2503	.250 ug/well .2504	.2505	.2506	.2507	.2508	.2509	.2510	
C	BL	.5001	.5002	.5003	.500 ug/well .5004	.5005	.5006	.5007	.5008	.5009	.5010	
D	BL	1.001	1.02	1.03	1.00 ug/well 1.04	1.05	1.06	1.07	1.08	1.09	1.10	
E												
F												
G												
H												

FIGURE 8

RESULTS

Plate 1 (1:1000 IgM Ab)

Plate 1	0.125 ug /well SRBC	0.25 ug /well SRBC	0.5 ug /well SRBC	1 ug /well SRBC
1:8	1.667	1.466	1.702	1.701
1:16	1.647	1.681	1.700	1.672
1:32	1.588	1.575	1.590	1.614
1:64	1.369	1.342	1.406	1.476
1:128	1.070	1.114	1.173	1.176
1:256	0.761	0.770	0.822	0.900
1:512	0.432	0.492	0.493	0.562
1:1024	0.220	0.280	0.277	0.296
1:2048	0.093	0.121	0.126	0.119
1:4096	0.055	0.058	0.067	0.055
1:8192	0.087	0.118	0.035	0.032



Conclusion. No discernable difference between concentrations of SRBC. Use 0.125 ug/well SRBC.

Masking Wells

If you find data in certain wells (outliers) that should be excluded from the calculations being performed, you can mask these wells. Select the well(s) to be masked and then click the Mask button in the Plate section tool bar or choose **Mask...** from the **Plate** menu. Masked wells are shown with an overlay of diagonal lines.

The masking function can be used as a "what if?" tool. For example, you could suppress or enable certain functional groups within the experiment. Suppose you have included a group blank in the template and want to see the data with and without the blank. Masking the group blank wells would suppress the blanking function; unmasking them would enable it again.

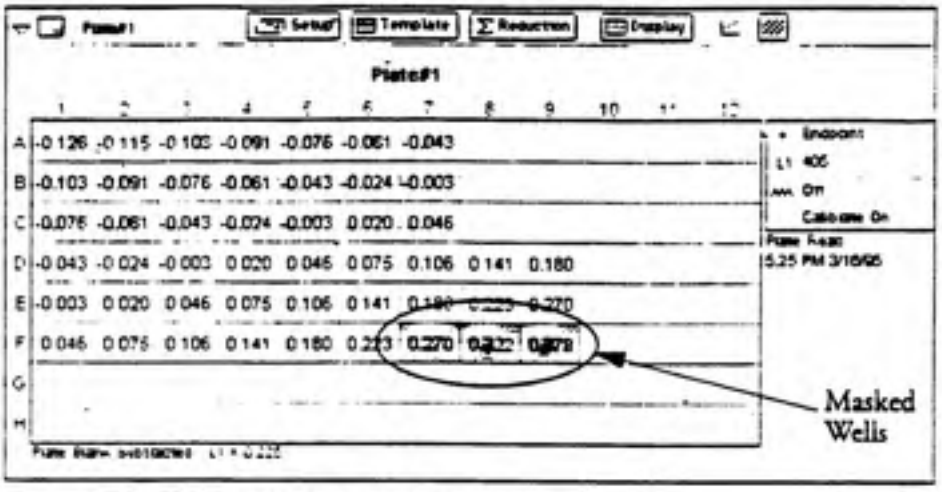


Figure 5.11: Masked Wells in the Plate Section

To remove masking from one or more wells, highlight the masked well(s) and click the Mask button in the Plate section tool bar or choose **Mask...** from the **Plate** menu again. The diagonal lines (and masking function) will be removed.

⚠ **CAUTION:** Formatting a disk will remove all existing data on it. Be sure that the disks you are using for backups do not contain any data you wish to keep.

5) A small dialog box will appear asking you to insert the source disk. Insert the SOFTmax PRO Program disk into the floppy drive.

Figure 2.7: Copy Disk—Insert Source Disk Dialog Box

Click the OK button or press Enter to continue with the copying process. A dialog box will appear showing the progress during the disk copy process.

6) When the contents of the source disk have been copied into memory, a dialog box will appear asking you to insert the destination disk (if you are using two disk drives, copying will simply continue). When copying is complete, the dialog box will disappear.

7) Follow steps 2 through 5 for the SOFTmax PRO Application Protocols disk. When you have copied both disks, put the originals away for safekeeping. Use the backup disks to install the software onto your hard disk.

Computer System Requirements

The following computer hardware and operating system software specifications are required to ensure proper operation of SOFTmax PRO.

Macintosh Computer

- Macintosh computer with 68040 processor (Quadra 610 or better)
- Operating software System 7.1 or greater
- 14-inch color monitor or larger
- 8 MB RAM (minimum)
- 80 MB hard disk drive
- 1.44 MB HD (high density) 3.5-inch floppy disk drive
- 8-pin DIN to 8-pin DIN straight-through serial cable (for SPECTRAmax) or 8-pin DIN to DB 25 straight-through modem cable (for Emax, Vmax, UVmax, or THERMOmax). Molecular Devices recommends using only high-quality, double-shielded cables.

4) In order to allow the Macintosh to automatically recognize protocol and data files of PC origin as SOFTmax PRO files, you need to enter the associated three-letter extensions that correspond to these files in the PC Exchange Add dialog box. The table below lists the type of file and its corresponding extension that should be entered in the dialog box.

Table 3.1: PC File Name Extensions for Use with PC Exchange

Type of File	3-Letter Extension
SOFTmax PRO Protocol File	.PPR
SOFTmax PRO Data File	.PDA
SOFTmax Protocol File	.PRO
SOFTmax Data File	.DAT

For example, if you will be working with SOFTmax PRO *protocol files* created on the PC, enter .PPR in the box under "Dos Suffix."

5) Then click the application icon (currently shown as "Not Specified" in the center of the box under "Application Program." From the menu choices, navigate to the folder where the SOFTmax PRO application resides. Highlighting this application will enable the Macintosh to start SOFTmax PRO and read the corresponding protocol file whenever you double-click a PC file bearing the ".PPR" extension. Clicking [OK] causes this dialog box to close and updates the earlier dialog box to show the extension and associated program you just entered.

6) Repeat steps 3 through 5 for other PC file name extensions with which you will be working.

Transferring Macintosh Files to the PC

Software is available for the PC that allows the insertion and reading of Macintosh 3-1/2-inch disks. Since it is not commonly in use, however, we are assuming that the transfer of files to the PC disk would normally be done using the Macintosh superdrive.

Macintosh computers (except for some early models) are equipped with software that allows you to "read" and transfer files from 3.5-inch PC disks. If you are using System 7.1, you must use Apple File Exchange or a third-party software program to enable you to transfer Macintosh files to PC floppy disks. Those of you using System 7.5 can use PC Exchange for this process, which makes transfer easier. The process of using PC Exchange is described below. Check your Macintosh documentation to learn how to transfer files using Apple File Exchange.

1) If you have not yet enabled PC Exchange on your Macintosh, select Control Panels from the Apple (🍎) menu. From within the Control Panels, choose the Extensions Manager and turn PC Exchange on by placing a checkmark next to it. Restart your computer—PC Exchange will be enabled.

AFC or PFC Assay for SRBC Immunized Animals
AFC (Antibody Forming Cell)
PFC (Plaque Forming Cell)

1. Scope of Application

The hemolytic plaque forming cell (PFC) or antibody forming cell (AFC) assay, which measures the primary IgM response to SRBC, can be performed using a number of techniques. The two most frequently used hemolytic plaque techniques employ either a mixed suspension of spleen cells, SRBC and complement added to a specially prepared microscope slide (Cunningham, 1965; Cunningham and Szenberg, 1968) or the addition of the mixed suspension of spleen cells, SRBC and complement to melted agar, which is then poured into a petri plate and allowed to solidify (Jerne and Nordon, 1963). The procedure given below is a modification of the Jerne and Nordin (1963) technique as described by Moller et al. (1973).

2. Prerequisites

2.1 Equipment and supplies

SRBC in Alsever's (Maximum 3 weeks past date drawn) (Colorado Serum)
Guinea pig complement (Gibco)
Purified Agar (Difco)
DEAE-Dextran (Pharmacia Chemical)
Earl's Balanced Salt Solution (EBSS 10 X) (Gibco)
RPMI (Gibco)
Gentamicin (Gibco)
FCS (fetal calf serum) (Hyclone)
Isoton (Coulter thur Krackeler)
Zapoglobin (Coulter thur Krackeler)
Trypan Blue 0.4% (Gibco)
120 ml Fleaker
9 ml tube with top
Hepes Buffer (1 M) (Gibco)
saline
microscope slide (25 x 75 mm)
cover glass (45 X 50 mm)
cover glass (22 X 22 mm)
100 mm petri dish (4/sample)
75 X 12 mm dilution tube (5 ml)
10 X 75 mm disposable tube (glass)
Surgical instruments
pasteur pipet 5 3/4"
pasteur pipet 9"
Eppendorf combitips, size 1.25, 5, 12.5, and 50 ml

pipet tips size 1-200 µl
Stomacher 80 bags 1/organ
Coulter counter
Coulter Diluter III
balance
counting vials
Stomacher 80
water bath
incubator
microscope
2 X 2 gauze (no cotton)
rubber bulbs
automatic pipet aid
ice
ice containers
racks and vial holders
Plaque viewer
centrifuge (IEC DPR-6000)
electronic diffcount
4C cell control (Coulter)

2.2 Training Requirements

New personnel will receive on the-job training from lab personnel familiar with this procedure. 4C is prepared from human blood, therefore required bloodborne pathogen training is a prerequisite for handling this material.

3. Cautionary notes or Special considerations

4C is prepared from human blood and is used as a QC standard for monitoring the Coulter counter. Since 4C may harbor bloodborne pathogens, appropriate protection (i.e., lab coat and disposable gloves) must be worn by personnel handling this material.

4. Procedure

4.1.1 Preparation of spleen cells.

1. Bleed immunized animal for serum to be used for HA (if you plan to run the assay).
   Rat: Kill rat by CO_2 anoxia followed by cervical dislocation. Rats were immunized iv with 0.5 ml of 2.5% SRBC (washed 3 times with saline and centrifuged 7 mins. at 2000 rpm or 1200 xg) 4 days prior to assay.
   Determine and record SRBC concentration of the 2.5% suspension using Coulter Counter on day of immunization. The 2.5% suspension of SRBC should be approximately $5X10^8$ SRBC/ml (i.e., $2.5x10^8$/rat). Remove the spleen, place in a 9 ml tube containing 5 ml of RPMI + 5% FCS + gentamicin. Use stock gentamicin (50 mg/ml) and add 0.1 ml/100 ml RPMI. Put into Stomacher 80 bag and disburse cells

from the spleen using the Stomacher 80. Remove cells from bag with a 9 ml pasteur pipet, put cells back in the same tube, then rinse bag with another 3 ml RPMI.
TOTAL 8 ml in the tube.

Mice: Kill the mice by CO_2 anoxia followed by cervical dislocation. Mice were immunized iv with 0.2 ml of 5.0% SRBC (washed 3 times with saline and centrifuged 7 mins at 2000 rpm or 1200 xg) 4 days prior to assay. Determine and record SRBC concentration of the 5% suspension using the Coulter Counter on the day of immunization. The 5% suspension of SRBC should be approximately $1x10^9$ SRBC/ml (i.e., $2x10^8$ SRBC/mouse). Remove the spleen, place in a 9 ml tube containing 4 ml of RPMI + 5% FCS + gentamicin (same concentration as for the rat). Put spleen into Stomacher 80 bag and disburse cells from the spleen using the Stomacher 80. Remove cells from the bag with a 9 ml pasteur pipet, put cells back into same tube, then rinse bag with another 1 ml of RPMI.
TOTAL 5 ml in the tube.

2. Invert the tube of cells, allow to settle (to remove clumps/trash) on ice for 10 minutes. Transfer to a clean 9 ml tube by pouring cells through a section of 2 X 2 gauze. This is the "stock" cell suspension.

3. Make dilutions of "stock" cell suspension for AFC/PFC assay. This dilution will depend on strain of animal or concentration of cells. Normal dilutions are 1:10, 1:20, 1:40 or 1:60. Make 2 dilutions for each spleen "stock" cell suspension for each animal.

4.1.2 **Spleen cell concentration.**

1. Turn Coulter counter on to warm up 10-15 minutes before you plan to count samples. Remove the 4C cell control from the refrigerator and warm to room temperature. Be sure to check that 4C control date has not expired. Roll the vial slowly between the palms of the hand and gently invert vial several times. For your protection wear disposable gloves at all times, **4C is prepared from human blood.** Gently remove rubber stopper from vial, make a WBC dilution on the Diluter III. Immediately replace top on the 4C and return to the refrigerator. Lyse RBC by adding 4 or 5 drops of Zapoglobin to the vial. Invert GENTLY, count on the Coulter counter. 4C is intended for monitoring the performance of the Coulter instrument. Check the Table of Expected Results, that comes with each shipment of 4C, to obtain expected value. When expected value is obtained record in notebook. Dispose of vials, gloves, wipes and etc in **Biohazard Container.** Be sure counter area is cleaned! Use Amphyl or PDI towelette (benzalkonium chloride antiseptic). If a spill occurs clean with Isolyser.

2. **Determine cell count:**
Remove cells from "stock" tube for counting by using the Coulter Diluter III (set on WBC) or pipet 40 µl of cell into coulter vial that contains 19.96 ml of Isoton. Lyse RBC with Zapoglobin and count on the Coulter counter. The results are given as #

cells $X10^6$/ml or E^6/ml.

3. **Determine percent viability:**
Put 10 µl of Trypan Blue (0.4%) on a microscope slide add 10 µl of "stock" cells. Cover with 22 X 22 cover glass and count 100 cells on the microscope. Record count on electronic diffcount. This percentage will be used later to calculate total viable cells/spleen. The spleen cells may be counted after AFC/PFC assay is set up, provided cells remain on ice.

4.1.3 Preparation of Complement: This should be done before day of assay.

1. Wash SRBC 3 times with saline. Remove supernate of last wash, put SRBC pellet into container of ice.

2. Reconstitute lyophilized guinea pig complement, stored in freezer (Gibco), with guinea pig complement restoring solution, stored in refrigerator (Gibco). Keep on ice until all lyophilized complement is in suspension. Absorb by adding 1 drop of SRBC pellet to complement solution. The 1 drop of SRBC is added to eliminate any non-specific lysis of SRBC by the complement. Keep on ice for 30 minutes. Gently swirl, put solution in a 50 ml centrifuge tube. Centrifuge 20 minutes at 2000 rpm, 1200xg to remove SRBC in the IEC, DPR 6000. The supernate is "stock" complement. Put 1.5 ml of absorbed complement in 5 ml tubes with tops. Wrap top with parafilm and store in freezer.

3. On day of assay remove a tube of "stock" complement, dilute 1:3 with RPMI. Keep on ice. This should be enough complement for an assay of 30 animals.

4.1.4. Preparation of SRBC:

1. Wash SRBC 3 times with saline. Centrifuge each wash in the IEC, DPR 6000, for 7 minutes at 2000 rpm, 1200xg. Resuspend to 40-50% with RPMI media. Keep on ice.

4.1.5. Preparation of Agar: This is done **First thing** on day of assay.

1. Set water bath temperature to 47°C.

2. Put warm water into a container to boil. Use gas burner to heat water.

3. Put 500 mg Purified Agar (Difco) into a fleaker. Add 90 ml of distilled water, 10 ml of EBSS (stock is 10 X =1:10 dilution) and add 5 ml of 1 M Hepes buffer. Cover the fleaker with aluminum foil.

4. Melt agar by placing fleaker in boiling water for 10 minutes. Add 1.6 ml of DEAE dextran. **Mix well.** The agar will become cloudy. Place in 47°C water bath.

DEAE dextran: 30 mg/ml in 0.9% NaCl adjust to pH 6.9 with 1 M Hepes buffer and filter. Store in refrigerator. This is stable about 6 months.

4.1.6. AFC\PFC Assay

1. Put 10 X 75 glass tubes into a 47°C water bath. Allow to warm at least 15 minutes then add 350 µl agar to each tube. 4 tubes/animal are needed.

2. Add 25 µl of 40-50% SRBC suspension to 8-10 tubes.

3. Add 100 µl of each cell dilution to each of 2 tubes. Start with lowest cell dilution.

4. Add 25 µl of complement.

5. After the addition of complement, vortex sample (try to avoid bubbles).

6. Pour into the center of a 100 mm petri dish, quickly cover with a 45 X 50 cover glass. Do not move plate for 10-15 minutes (this is to allow the agar to gel). Combine top/bottom of petri dish, may be stacked and placed in 37°C incubator for 3 hours.

7. Count all plaques on the Plaque viewer using an electronic diffcount.

4.2 Quality Control

Petri dishes are to be labeled with sample # and dilution #. There will be 2 dishes/dilution and a total of 4 dishes/spleen. Count all samples.
Dishes to be used are Corning #25020, **BOTTOM ONLY**
These have been tested and proven to produce reproducible plates.
4C is used for monitoring the Coulter counter. Control animals are used in each experiment. SRBC should not be over 3 weeks old.

4.3 Evaluation Criteria

The enumeration of plaque forming cells present in a lawn of SRBC, complement and agar. A quantitative and subjective count should be done on all 4 plates. The plaque forming cells may appear in various sizes, consistent counting procedure is critical. Use a plaque viewer for evaluation.

4.4 Corrective Action

N/A

4.5 Recordkeeping

All data and information are recorded on PFC data sheet. Data necessary for calculations are spleen cell count, viability count and 4 PFC counts (2 counts for each spleen

cell dilution). Information required on the data sheet includes the name of the experiment, the date of assay and the initials of person or persons recording data. Computer-generated calculations, both hard copy and data files, are maintained as part of the permanent record of each experiment. Data files are stored on a back up floppy disk and hard copy print outs of data are place in a 3 ring binder. Data files and the hard copy of data are identified by an identical 8 character code.

4.6 Calculations

Data (i.e., cell count, viability and PFC counts) are entered into a Lotus file (Tucker) which generates PFC/10^6 and PFC/spleen for each animal. These data are analyzed by one-way analysis of variance, with post hoc analysis using Dunnett's multiple comparison t-test or the Student-Newman-Keuls multiple range test using

RS-1. A value of $p<0.05$ is considered significant.

5.0 Quality Control Rationale

4C is used to insure that the Coulter counter is working properly. Duplicate plates for each dilution are used to assess reproducibility within each dilution for the counted plaques. Incubator temperature and CO_2 are monitored. Temperature is monitored for the water bath. The pH of PBS is checked using the pH meter. The pH meter, centrifuge and balance are checked every 6 months by a contract auditor.

6.0 References

Cunningham, A.J. (1965). A method of increased sensitivity for detecting single antibody-forming cells. Nature 20:1106-1107.

Cunningham, A.J., and A. Szenberg (1968). Further improvements in the plaque technique for detecting single antibody-forming cells. Immunology 14:599-602.

Jerne, N.K., and A.A. Nordon (1963). Plaque formation in agar by single antibody producing cells. Science 146:405.

Moller, E., W.W. Bullock, and O. Makela (1973). Affinity of T and B lymphocyte receptors for hapten determinants. Eur. J. Immunol. 3:172-179.